PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/85, A61K 48/00, C12N 15/11

A1

(11) International Publication Number: **WO 98/40506**

(43) International Publication Date: 17 September 1998 (17.09.98)

(21) International Application Number: PCT/EP98/01363

(22) International Filing Date: 9 March 1998 (09.03.98)

(30) Priority Data:
97103916.9 8 March 1997 (08.03.97) EP
(34) Countries for which the regional or international application was filed: DE et al.

(71) Applicant *(for all designated States except US)*: BOEHRINGER MANNHEIM GMBH [DE/DE]; Sandhofer Strasse 116, D-68305 Mannheim (DE).

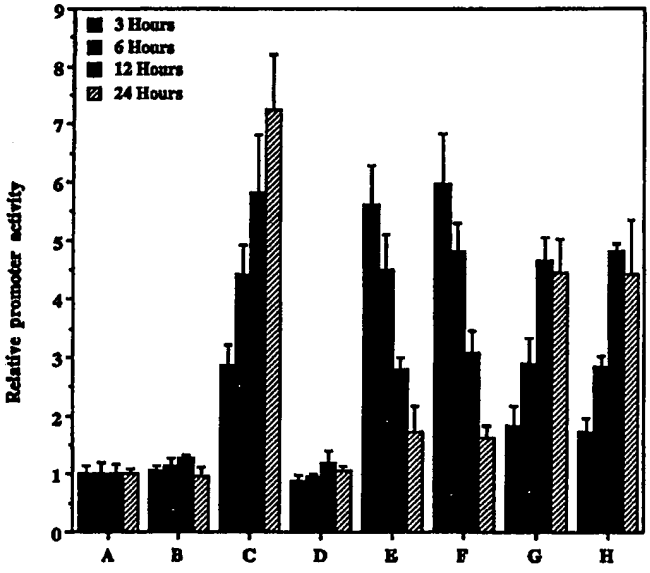
(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: WHITEHEAD, Alexander [GB/US]; 54 Briar Road, Strafford, PA 19087 (US). UHLAR, Clarissa [US/US]; 2509 Waverly Street, Philadelphia, PA 19146 (US).

(74) Common Representative: BOEHRINGER MANNHEIM GMBH; Werk Penzberg, Patentabteilung (RE-TB), Postfach 11 52, D-82372 Penzberg (DE).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: USE OF AN ACUTE PHASE SERUM AMYLOID A GENE PROMOTER (A-SAA PROMOTER) IN THE TREATMENT OF DISEASES IN MAMMALS



(57) Abstract

A gene therapy agent containing a vector with a gene coding for a therapeutically useful protein for the treatment of a mammalian disease wherein a) an inflammatory response consisting of a local in vivo production of inflammatory mediators like IL-6 and/or IL-1β is induced at a predetermined locus or systemically by said agent; b) the gene coding for said protein is under the control of an acute phase serum amyloid A gene promoter, and c) the expression of said gene is induced by said inflammatory mediators, is useful for the treatment of mammalian diseases, especially of inflammatory diseases.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

Use of an acute phase serum amyloid A gene promoter (A-SAA promoter) in the treatment of diseases in mammals

The invention concerns the use of an acute phase serum amyloid gene promoter in the treatment of diseases in mammals, especially in the treatment of inflammatory diseases and in gene therapy.

Inflammation is the immediate, innate response which has evolved to enable an organism to counteract challenges such as tissue injury, infection and trauma (Kushner, 1982). The first set of reactions elicited form the acute phase response which is mediated initially by the release, primarily by activated macrophages, of cytokines at the site of tissue damage (Steel and Whitehead, 1994; Baumann and Gauldie, 1994). These cytokines, particularly interleukin-1 (IL-1) and tumour necrosis factor (TNF), initiate an inflammatory mediator cascade which is characterized locally by vascular changes, erythema, swelling and pain, and systemically by physiological changes including fever and metabolic alterations. Although it has evolved to play a beneficial role in the short term, maintenance of the acute phase response in cases of chronic inflammation may have dramatic, negative consequences. One of the primary changes that occurs during the acute phase response is the alteration of the synthetic profile of the liver which produces increased amounts of acute phase reactants (APRs) (Fey and Gauldie, 1989). This is triggered by pro-inflammatory molecules (e.g. IL-1 and TNF) which act via receptor-mediated signal transduction events to activate transcription factors (e.g. NF-kB). These in turn bind to specific regions in the promoters of APR genes and induce transcription. The promoters of a subset of these genes, those encoding the major APRs (in humans the acute phase serum amyloid As [A-SAAs] and C-reactive protein [CRP]), are extremely responsive to such signals causing them to be massively induced during the acute phase response (Kushner, 1982; Kushner and Mackiewicz, 1987; Fey and Gauldie, 1989). Consequently major APR promoters have the potential to be used as indicators of the ability of naturally occurring and synthetic molecules to act as pro- and anti-inflammatory reagents. They also could be adapted to drive the synthesis of products of heterologous genes, both in vivo for gene therapy and in vitro for the large scale synthesis of recombinant proteins.

Human A-SAA is the product of two genes, SAA1 and SAA2, which are almost identical with respect to their organization, sequence and mode of expression (Sipe, 1985; Woo, 1987; Dwulet, 1988; Beach, 1992). A-SAA levels increase by as much as 1000-fold during the acute phase response (Hoffman and Benditt, 1982; Marhaug, 1983). Although this dramatic induction by inflammatory stimuli indicates an important protective role for A-SAA in the short term, the long term synthesis of high levels of A-SAA in chronic inflammation has negative clinical consequences. A-SAA is the serum precursor of amyloid A which is the principal constituent of the insoluble fibrils deposited in organs and tissues in the progressive, fatal condition secondary or reactive systemic amyloidosis (reviewed by Steel and Whitehead, 1994). The massive inductive capacity of A-SAA gene transcription, together with the clinical importance of A-SAA in the context of chronic inflammation makes the A-SAA promoters ideal candidates for use in rapid and sensitive assays for assessing the agonist and antagonist activities of known and putative mediators of inflammation. For the same reasons the way in which the A-SAA promoters respond to various inflammatory mediators and inhibitors is itself of intrinsic importance.

Acute phase serum amyloid A (A-SAA) is a major APR whose concentration increases by up to 1000 fold during the acute phase response (Hoffman and Benditt, 1982; Marhaug, 1983). It is the product of two genes in humans, SAA1 and SAA2 (Sipe et al., 1985; Woo et al., 1987; Dwulet et al., 1988; Beach et al., 1992). IL-1ß and TNFα are cytokines which act via similar multiple signalling pathways in cells to transcriptionally activate target genes (reviewed by Baumann and Gauldie, 1994). These include type-1 APRs such as human A-SAA and C-reactive protein (CRP). IL-1ß and TNFα may act synergistically when combined with IL-6 to increase type-1 APR gene transcription (Baumann and Gauldie, 1990).

The promoters of A-SAA genes, preferably of type-1 genes, are intrinsically interesting for studies of complex cytokine-driven gene expression, and are attractive candidates for the generation of promoter-reporter constructs of great sensitivity for use in assessing the pro- and anti-inflammatory properties of natural and synthetic molecules.

Summary of the invention

The invention comprises the use of a gene therapy agent which contains a vector with a gene coding for a therapeutically useful protein for the treatment of a mammalian disease,

a) wherein an inflammatory response consisting of a local in vivo production of inflammatory mediators like IL-6 and/or IL-1ß is induced at a predetermined locus or systemically by said agent;

b) the gene coding for said protein is under the control of the human acute phase serum amyloid A gene promoter SAA1 or SAA2, and

c) the expression of said gene is induced by said inflammatory mediators.

In a preferred embodiment of the invention the gene therapy vector is supplied in a single local bolus dose.

The invention further comprises a process for the in vitro production of a predetermined recombinant protein in eukaryotic cells by expression of a gene with a nucleic acid sequence encoding said protein under the control of a regulatory sequence which contains the promoter region of a human acute phase serum amyloid A gene, and co-expression of transcription factors in said cells, which activate said promoter, and recovering the protein from the host cell culture.

A further preferred embodiment of the invention is the use of a gene therapy vector for the treatment of an inflammatory disease, wherein

a) said vector contains a nucleic acid which codes for a cytokine-inhibiting protein, which gene is under the control of a human acute phase serum amyloid A promoter;

b) said nucleic acid is expressed at the site of said inflammatory disease, and

c) said protein inhibits the inflammatory disease by inhibiting the action of cytokines on cells.

The invention further comprises methods of manufacturing therapeutic agents using the above-mentioned gene therapy vectors.

The invention further comprises new expression control regions (promoters) which are the control regions of A-SAA genes, whereby the A-SAA1 promoter is preferred.

Detailed description of the invention

The A-SAA promoter is highly sensitive to cytokine induction during local or systemic inflammation. It is therefore particularly suitable for incorporation into constructs designed to drive the synthesis of therapeutic natural or synthetic molecules that may be effective in eliminating or controlling diseases with an inflammatory component (e.g., rheumatoid arthritis). A particular advantage is that when the inflammation associated with disease has been controlled through the action of the expressed agent the further expression of the agent will cease. Expression may therefore be induced only when required.

A gene therapy agent according to the invention is understood to mean a therapeutic composition which contains a vector with a gene coding for a therapeutically useful protein for the treatment of a mammalian disease. Such a therapeutic composition preferably contains the vector together with a non-viral delivery system or as a retroviral vector. In such cases, the delivery system or the retroviral vector per se or the expressed gene will cause a local or systemic inflammatory response. Based on this and on such an immune (i.e., inflammatory) response, the A-SAA promoter may also be applied to gene therapy protocols aimed at controlling diseases that do not have a primary inflammatory component. In such cases cytokine mobilization caused by the administration of the delivery/targeting vehicle would lead to the A-SAA promoter driven production of the therapeutic agent at up to at least a hundred times that which can be achieved using currently available promoters.

Gene therapy of somatic cells can be accomplished by using, e.g., retroviral vectors, other viral vectors or by non-viral gene transfer (for clarity, cf. Friedman, T., Science 244 (1989) 1275; Morgan 1993, RAC Data Management Report, June 1993).

Vector systems suitable for gene therapy are, for instance, retroviruses (Mulligan, R.C., (1991) in Nobel Symposium 8: Etiology of human disease at the DNA level (Lindsten, J., and Pattersun, eds.) 143-189, Raven Press), adeno-associated virus (McLughlin, J. Virol. 92 (1988) 1963), Vaccinia virus (Moss et al., Ann. Rev. Immunol. 5 (1987) 305), bovine papilloma virus (Rasmussen et al., Methods Enzymol. 139 (1986) 642) or viruses from the group of the Herpes viruses, such as Epstein-Barr virus (Margolskee et al., Mol. Cell. Biol. 8 (1988) 2937) or Herpes simplex virus.

There are also known non-viral delivery systems. For this, usually "nude" nucleic acid, preferably DNA, is used, or nucleic acid together with an auxiliary agent, such as, e.g.,

transfer reagents (liposomes, dendromers, polylysine transferrin conjugates (Felgner et al., Proc. Natl. Acad. Sci. USA 84 (1987) 7413).

"Inflammatory response" means a local or systemic in vivo production of inflammatory mediators like IL-6 and/or IL-1ß.

In response to a local challenge (e.g., infection, trauma, tissue necrosis, burns, etc. and chemical agents such as those used as delivery systems in gene therapy), inflammatory effector cells, especially macrophages, release a range of pro-inflammatory mediator molecules. Amongst the most important of these are IL-1. IL-1 in turn generates a cytokine cascade leading to the recruitment of additional cells and tissues into the inflammatory process and the consequent production of additional pro-inflammatory cytokines like IL-6. This process may become systemic and, in the absence of continued challenge, is ultimately controlled and reversed by the release of hormones such as glucocorticoids and cytokine antagonists such as IL-1ra. In chronic inflammatory conditions the pro-inflammatory signals remain predominant giving rise to various disease pathologies (e.g., joint degeneration in rheumatoid arthritis).

Inflammatory inhibitors are inhibitors of these mediators like antibodies or receptor molecules binding said mediators.

"Therapeutically useful proteins" are, in particular, proteins that are useful in the treatment of cancer and inflammatory diseases. Especially preferred are cytokines with a demonstrated biological effect such as IL-2 which activates various T-cell subsets and thereby enhances anti-tumour function, and IL-1ra which acts as a direct antagonist of the major (initiating) pro-inflammatory cytokine IL-1. Additional "therapeutically useful proteins" include (but are not limited to) cytokines such as IL-4 and IL-10, and soluble cytokine receptors such as those for IL-1 and TNF.

"Desired proteins" include the therapeutically useful proteins as well as other proteins which can be produced, advantageously, in vitro, such as, for instance, interferons, erythropoietin, thrombopoietin and other blood proteins, as well as thrombolytics, such as plasminogen activators.

A cytokine responsive construct, pGL2-SAA2pt, was generated by cloning the acute phase promoter of human serum amyloid A2 (SAA2) upstream of a luciferase reporter gene. The

construct responds to the inflammatory mediators MoCM, IL-1ß, TNFα, and IL-6 in a manner that closely mimics the response of the endogenous SAA2 gene to such stimuli: i.e. single treatments induce transcriptional activation by IL-1ß and TNFα to a greater extent than by IL-6 at 12-24 hours. However, timecourse experiments show that the kinetics of induction generated by IL-1ß and TNFα are quite distinct from that generated by IL-6, IL-6 having a much greater effect at 3-6 hours. IL-1ß and TNFα synergize with IL-6 to give a 10 fold increase or more in transcriptional readout over single cytokine treatments. The kinetics of this synergistic response resembles that generated by IL-6 alone. The IL-1 receptor antagonist, hIL-1ra, can specifically block the IL-1ß driven transcriptional activation of pGL2-SAA2pt, but not that driven by TNFα or IL-6. Furthermore, in synergistic cytokine combinations, it blocks only the IL-1ß driven component indicating that the effect is biological and not attributable to toxicity. Consequently assays utilizing pGL2-SAA2pt will be useful both for the investigation of the kinetics of inflammatory signalling in a cytokine specific manner, and for the evaluation of the pro- and anti-inflammatory properties of novel natural and synthetic molecules.

By "SAA promoter" according to the invention a promoter which has the function of an SAA promoter and is essentially identical to the sequence of the SAA1 and/or SAA2 promoter is understood. In view of the fact that there is only a 10% difference in sequence between the SAA1 promoter and the SAA2 promoter, it is obvious that such a difference in sequence does not have a substantial influence on the functionality of the SAA promoter, and that such variations therefore are not essential for the function.

The SAA2 gene sequence was described by Steel et al., 1993. An SAA2 genomic sequence was published by Woo et al. in 1987. There is a second hyperinducible SAA gene, SAA1. The SAA4 gene locus has been published in 1991 by Betts et al.. No analysis of the SAA1 promoter has been done. The SAA1 and SAA2 sequences are approximately 91% identical and tissue culture studies and in vivo studies of the equivalent genes in other species indicate that they are co-ordinately regulated.

An SAA promoter according to the invention is described in Fig. 1 and in SEQ ID NO: 1. Also preferred are promoters which are coded by DNA sequences which hybridize with SEQ ID NO: 1 under stringent conditions and have the ability to act as an expression control sequence.

The phrase "hybridize under stringent conditions" means that two nucleic acid fragments are capable of hybridization to one another under standard hybridization conditions described in Sambrook et al., "Expression of cloned genes in E.coli" in Molecular cloning: a laboratory manual (1989), Cold Spring Harbor Laboratory Press, New York, USA, 9.47-9.63 and 11.45-11.61. More specifically, "stringent conditions" as used herein refers to hybridization in 6.0 x SSC at about 45°C followed by a wash of 2.0 x SSC at 50°C. For a selection of the stringency the salt concentration in the wash step can be selected, for example, from about 2.0 x SSC at 50°C for low stringency to about 0.2 x SSC at 50°C for high stringency. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions, about 65°C.

The invention therefore further comprises an isolated first nucleic acid molecule which codes for a regulatory sequence for the expression of a second nucleic acid coding for a protein, the expression control of which is regulated by inflammatory mediators and inhibitors, wherein said first nucleic acid molecule is selected from the group consisting of

(i) the expression control region shown in SEQ ID NO:1 or the complementary sequence, or
(ii) an expression control sequence which hybridizes with SEQ ID NO:1 or the complementary sequence under stringent conditions.

The invention further relates to the use of the gene therapy vectors according to the invention for the treatment of inflammatory diseases. The vectors of the invention are suitable for a specific treatment of inflammatory diseases since the genes which are under the control of the SAA promoter are expressed essentially only in those cells, or in the immediate surroundings thereof, in which inflammatory processes are taking place. In such case, the genes used are genes coding for proteins which have an antiinflammatory effect, including those that inhibit cytokines.

Cytokines are inhibited according to the invention as has been demonstrated for the interleukin 1 receptor antagonist. The IL-1 receptor antagonist would be preferred for developing anti-inflammatory procedures based on expression driven by the SAA promoter. Other useful inhibitors, the expression of which could be driven by the SAA promoter, include the soluble type 2 IL-1 receptor and the soluble TNF receptor. Anti-inflammatory cytokines (e.g., IL-4 and IL-10) are additional candidates.

According to the invention it is preferred to use, under the control of the SAA promoter, the gene encoding the thymidine kinase of Herpes simplex virus type I (HSV-TK). Certain guanosine analogues, such as Acyclovir, Ganciclovir, are specific substrates for HSV-TK which catalyzes their phosphorylation to monophosphate. A further useful gene is the bacterial gene for a cytosine deaminase which confers in mammalian cells lethal sensitivity to 5-fluorocytosine (Mullen, C.A., Proc. Natl. Acad. Sci. USA 89 (1992) 33-37).

A "lethal polypeptide" is capable of killing the cell, for example, upon its own induction via the SAA promoter or upon addition of a substrate for a lethal polypeptide, which substrate is converted by the lethal polypeptide to a molecule that is toxic for the cell.

By the term "under the control" it is meant that the promoter is located in a position relative to that of the DNA encoding the desired polypeptide that allows the promoter to efficiently direct transcription of the structural gene for the desired polypeptide.

The invention demonstrates that the SAA promoter responds synergistically to combinations of cytokines (i.e., IL-1 plus IL-6; TNF plus IL-6; and monocyte conditioned medium, a crude mixture of pro-inflammatory cytokines) over long periods following stimulus. Inductions of greater than 100-fold and inductions as high as 200-fold can be found. These are quantitatively the most dramatic inductions of promoter activity for terminally differentiated human cells. The use of luciferase, which has a relatively short half life (in contrast to the more commonly used CAT reporter, which gives a measure of accumulated product that may have been synthesized over time or have persisted since the beginning of the time-course of the analysis), permitted the examination of the inductive capacity of the promoter at particular timepoints following stimulus. The invention shows that the massive induction (i.e., high level promoter activity) can be sustained for at least 48 hours.

The invention describes also the generation of a cytokine-responsive construct (e.g., pGL2-SAA2pt, containing 1.2Kb of the SAA2 promoter) fused to a luciferase reporter gene. The construct may be used to study the kinetics of the promoter response to the inflammatory cytokines IL-1ß, TNFα and IL-6, and establish that the inductive effect of either IL-1ß or TNFα is at its maximum much later than the time at which maximum induction can be achieved with IL-6. In addition, the use of the construct in cell culture for inflammatory response assays establishes the effectiveness of the recombinant human IL-1 receptor antagonist (hIL-1ra) in specifically eliminating the IL-1ß component of the in vitro acute phase luciferase readout.

The invention is concerned further with a process for the production of recombinant proteins in eukaryotic cells, especially in mammalian cells. The so far known processes for the production of recombinant proteins in mammalian cells have a rate of induction that may well need to be improved. Surprisingly, it has been found that the acute phase serum amyloid A gene promoter is inducible at a considerably higher rate than the hitherto known systems when there is a coexpression of a transcription factor in the host cells.

Preferred transcription factors that could be used in conjunction with the SAA promoter are NFkB, CEBP/NF-IL6 and also others such as YY1, SAF and AP1.

Abbreviations: APRs, acute phase reactants; SAA, serum amyloid A; A-SAA, acute phase serum amyloid A; hIL-1ra, human interleukin-1 receptor antagonist; IL-1, interleukin-1; IL-6, interleukin-6; TNF, tumour necrosis factor; MoCM, monocyte conditioned medium.

A further object of the invention is a translation control element within the SAA2 5'-UTR that plays a crucial role in modulating A-SAA production. This element is a cell- and/or tissue-specific translational enhancer. Its efficiency could be mediated by an intracellular factor that is activated or synthesized de novo after cytokine treatment. The sequence of this enhancer element is shown in SEQ ID NO:4. Preferably, the enhancer is used in conjunction with the SAA promotor (i.e., downstream of the promotor and upstream of the gene encoding the product of interest). It is further named "SAA enhancer".

By "SAA enhancer" according to the invention there is understood an enhancer which has the function of an SAA enhancer and is essentially identical to SEQ ID NO:4. Also preferred are enhancers with DNA sequences which hybridize with SEQ ID NO:4 under stringent conditions and have the ability to act as an enhancer element.

Therefore, a further object of the invention is an isolated nucleic acid molecule which codes for an enhancer element for the expression of a nucleic acid coding for a protein, said enhancer element being selected from the group consisting of

(i) the enhancer element shown in SEQ ID NO:4 or the complementary sequence or
(ii) an enhancer element which hybridizes with SEQ ID NO:4 or the complementary sequence under stringent conditions.

The invention further comprises an expression vector containing an SAA promoter according to the invention and/or an SAA enhancer according to the invention and a sequence coding for a desired protein. Such a vector can be used according to the invention as a gene therapy vector for the treatment of a mammalian, preferably an inflammatory disease. It is also preferred to use such vectors containing the SAA enhancer according to the invention for the in vitro production of a predetermined recombinant protein in eukaryotic cells.

The following examples, references, sequence listing and drawing are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Figure Legends

Figure 1 (a) Nucleotide sequence of the upstream region of SAA2. 20 bp of the first exon of SAA2 included in the construct are in bold and the transcription start site is indicated under +1. The TATA box is double underlined, a consensus NF-kB site (-91 to -82) and an NF-IL6 site (-183 to -173) are singly underlined.
(b) The acute phase responsive construct pGL2-SAA2pt. The construct contains 1196bp of the SAA2 upstream region and 22 bp of the first exon (SAA2 prom) cloned into the multiple cloning site of pGL2-Basic (Promega) between MluI and XhoI upstream of the luciferase gene (luc). Ampr, ampicillin resistance; f1 ori, f1 origin of replication.

Figure 2 Induction of pGL2-SAA2pt in response to single cytokine and antagonist treatments. Transiently transfected human HepG2 hepatoma cells were harvested following various cytokine treatments (IL-1ß, TNFα and IL-6 at 10 ng/ml and hIL-1ra at 1 µg/ml) for 3, 6, 12 and 24h (see figure key) and luciferase and ß-galactosidase assays performed. Each treatment was performed in triplicate at each timepoint. Transfection efficiency was controlled for by calculating luciferase/ß-galactosidase ratios and the relative promoter activity normalised against untreated (medium only) cells is plotted.

Treatments: A- Medium only; B- hIL-1ra; C- IL-1ß; D- IL-1ß+hIL-1ra; E- IL-6; F- IL-6+hIL-1ra; G- TNFα; H- TNFα+hIL-1ra.

Figure 3 Induction of pGL2-SAA2pt in response to combination agonist and antagonist treatments. Transiently transfected human HepG2 hepatoma cells were harvested following various cytokine treatments (MoCM 1/20 dilution; IL-1ß, TNFα and IL-6 at 10 ng/ml; hIL-1ra at 1 g/ml) for 3, 6, 12 and 24h (see figure key) and luciferase and ß-galactosidase assays performed. Each treatment was performed in triplicate at each timepoint. Transfection efficiency was controlled for by calculating luciferase/ß-galactosidase ratios and the relative promoter activity normalised against untreated (medium only) cells is plotted. Treatments: A- Medium only; B- hIL-1ra; C- MoCM; D- MoCM+hIL-1ra; E- IL-1ß+IL-6; F- IL-1ß+IL-6+hIL-1ra; G- IL-6; H- TNFα+IL-6; I- TNFα+IL-6+hIL-1ra.

Figure 4.1 SAA2 promoter driven constructs used to investigate the SAA2 5'-UTR (structural elements not drawn to scale).

Figure 4.2 Aliquotting of cells transfected with SAA2 promoter driven luciferase constructs onto 6-well plates. The transfected cells were treated with 10 ng/ml IL-1ß + 10 ng/ml IL-6 and harvested at 0, 1.5, 3, 6, 12 and 24 hours (diagram shows one timepoint).

Figure 4.3 HepG2 cells transfected with the Sense 5'/Luc 3', Anti 5'/Luc 3', Anti Koz 5'/Luc 3' and Luc 5'/Luc 3' constructs treated with 10 ng/ml IL-1ß + 10 ng/ml IL-6.

Figure 4.4 HepG2 cells transfected with the Sense 5'/Luc 3', Anti 5'/Luc 3', Anti Koz 5'/Luc 3' constructs and treated with 10 ng/ml IL-6.

Figure 4.5 HepG2 cells transfected with the Sense 5'/Luc 3', anti 5'/Luc 3', Anti Koz 5'/Luc 3' constructs and treated with 10 ng/ml IL-1ß.

Figure 4.6 Principles of S1 nuclease protection assay.

Figure 4.7 S1 nuclease protection assay oligos.

Figure 4.8 S1 nuclease protection assay on RNA harvested from cells transfected with the Sense 5'/Luc 3' and Anti Koz 5'/Luc 3' constructs and treated with 10 ng/ml IL-1ß + IL-6.

Figure 4.9 (a) Levels of chimeric luciferase mRNA transcribed from the Sense 5'/Luc 3' and Anti 5'/Luc 3' constructs in HepG2 cells treated with 10 ng/ml IL-1ß + 10 ng/ml IL-6.

Figure 4.9 (b) Luc/ß-gal values from HepG2 cells transfected with the Sense 5'/Luc 3' and Anti 5'/Luc 3' constructs and treated with 10 ng/ml IL-1ß + 10 ng/ml IL-6.

Example 1

Generation of the pGL2-SAA2pt construct and description of reagents and assays whereby its characteristics may be determined.

Monocyte-Conditioned Medium (MoCM) Mononuclear cells were separated from whole human blood using a Ficoll-Paque gradient (Pharmacia Fine Chemicals, Milton Keynes, UK), resuspended in supplemented DMEM medium containing 10% (v/v) autologous human serum and allowed to adhere to a 100 mm tissue culture dish for 5h at 37°C. Non-adherent cells were removed and the remaining cells were washed three times with PBS (Gibco/BRL, Paisley, Scotland, UK). Adherent cells were stimulated for 48 h at 37°C by incubation in 10 ml fresh medium with 10 µg/ml E.coli lipopolysaccharide (10 endotoxin units/µg; Sigma, Dorset, UK) and MoCM was harvested.

Recombinant human IL-1ß having a specific activity 1.81×10^7 units/mg by ELISA was used. Recombinant TNFα having an activity of 2×10^7 L929 cytotoxicity assay units/mg was used. Recombinant human IL-6 having an activity of 5.1×10^5 T10 proliferation assay units/mg was used.

Construction of the pGL2-SAA2pt plasmid:

1196 bp of the promoter region and 22 bp of the first exon of the human acute phase SAA2 gene was amplified by PCR with the introduction of MluI and XhoI restriction sites at the 5'- and 3'-ends respectively (5'-oligonucleotide: 5'-AAGAATTCACGCGTCCATGCATGTTGCGGCCGCTTGGCCATCCTTTACTTCCT-3' (SEQ ID NO:2); 3'-oligonucleotide: 5'-TTGAATTCCTCGAGCAGGTACCATACA-TATGTAGCTGAGCT-GCGGGTCC-3') (SEQ ID NO:3). The PCR product was subsequently cloned into the multiple-cloning site of the pGL2-Basic vector (Promega, Madison, WI, USA) which is located upstream of a luciferase reporter gene. The correct orientation and integrity of the cloned fragment was verified by gel electrophoresis and DNA sequencing.

Transient transfections:

HepG2 cells (European Collection of Animal Cell Cultures, Porton Down, Salisbury, UK) were grown to confluence in complete medium (Dulbecco's MEM, 10% (v/v) fetal bovine serum, 0.5x MEM non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine; Gibco/BRL). All solutions and medium used were at 37°C. Cells from each T180 flask (Falcon, Becton Dickinson, Cowley, Oxford, UK) were resuspended in 500 µl incomplete medium, split between two Invitrogen electroporation cuvettes (R&D Systems, Abingdon, Oxford) and, following the addition of 10 µg each of pGL2-SAA2pt and pMJH.20 RSV (a transfection efficiency control kindly provided by Dr. Mary Weiss, Pasteur Institute, Paris) in 10 µl of H_2O, were electroporated at 250 V, 25 W and 25 mA. In a typical experiment cells from 9 cuvettes were pooled in 168 ml complete medium, plated at 1 ml/well in 6 well Falcon plates and incubated at 37°C, 5% CO_2 for 24 h. Cells were treated with fresh medium (1.5 ml/well) containing the appropriate inflammatory mediator(s) and incubated for 3, 6, 12 and 24 h. MoCM was added at 1/20 dilution; IL-1ß, IL-6 and TNFα at 10 ng/ml, and hIL-1ra at 1 µg/ml. Each treatment was performed in triplicate at each timepoint.

Luciferase and ß-galactosidase assays:

Following treatment cells were washed twice with PBS (Gibco/BRL) and incubated for 15 min. at room temperature in 0.5 ml 1x reporter lysis buffer (Promega). Cell lysates were harvested and 100 µl were mixed with 100 µl of luciferase assay buffer [20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 µM coenzyme A (Sigma), 470 µM luciferin (Sigma) and 530 µM ATP] at room temperature. For each individual lysate three consecutive luminometer (Turner Designs, Sunnyvale, CA, USA) readings were taken after 45 sec and subsequently averaged. 150 µl

cell lysate was mixed with 150 µl 2x ß-galactosidase buffer (0.12 M Na_2HPO_4, 0.08 M NaH_2PO_4, 2 mM $MgCl_2$, 100 mM ß-mercaptoethanol, 1.33 mg/ml ONPG) and incubated for 3-4 h at 37°C. Reactions were terminated by the addition of 500 µl 1 M Na_2CO_3 and the absorbance read at 420nm. The mean proportional increase in luciferase readout relative to the untreated control +/- SD was calculated for each triplicated treatment group at each time.

Example 2

Introduction of pGL2-SAA2pt into human hepatoma cells by transfection

A construct, pGL2-SAA2pt, which contains 1196 bp of the SAA2 promoter and 22 bp of exon 1 (Figure 1a) upstream of a luciferase reporter gene (Figure 1b) was synthesized. This was used to transiently transfect a human hepatoma cell line as SAA2 is primarily synthesized in the liver during the acute phase response. HepG2 cells have been used in studies on SAA expression as they respond to various inflammatory mediators including IL-1ß, TNFα and IL-6 (Steel et al., 1996; Betts et al., 1993); the HepG2 line can also be electroporated more efficiently than Hep3B hepatoma cells. Transient transfection was chosen in preference to the generation of stably transfected lines as hepatoma cell lines can change phenotypically with increasing passage number, i.e. transient transfection performed over time using aliquots of early passage cells grown in bulk produced qualitatively consistent results between experiments. Preliminary titration experiments in which transfected HepG2 cells were treated with 5, 10 and 25 ng/ml of either IL-1ß or IL-6 showed no significant difference between the three concentrations (data not shown). Following a 24 h recovery period cells were treated for 3, 6, 12 and 24 h with medium only as a basal level control; MoCM; and recombinant IL-1ß, IL-6, and TNFα, alone and in various combinations. The effectiveness of the construct in mimicking the documented dramatic response of the endogenous SAA2 gene to such stimuli was determined. Recombinant hIL-1ra was also used together with the pro-inflammatory cytokine treatments to evaluate its effectiveness in reducing/eliminating the luciferase readout from the SAA2 promoter that is driven by the above stimuli.

Example 3

Induction of pGL2-SAA2pt by single inflammatory agonists in the presence or absence of hIL-1ra

To demonstrate the ability of the construct, pGL2-SAA2pt, to respond to known pro-inflammatory mediators, individual treatments of transiently transfected cells with IL-1ß,

TNFα, and IL-6 were performed in triplicate. Figure 2 shows the amounts of luciferase activity produced by cells treated with various single cytokine agonists and/or hIL-1ra relative to that produced by untreated cells (medium only, Fig.2:A). Treatment with IL-1ß alone results in a 2.9 fold increase in SAA2 promoter driven transcription at 3 h (Fig.2:C). The level of induction increases at each subsequent timepoint to reach approximately 7.2 fold at 24 h. TNFα alone generates a similar transcriptional profile (Fig.2:G) though the fold induction of luciferase readout is slightly lower at each timepoint and there is no further increase in the level of induction achieved between the 12 and 24h timepoints. In contrast to IL-1ß and TNFα, IL-6 alone (Fig.2:E) has a more dramatic effect on SAA2 promoter driven transcription at the early timepoints: a 5.5 to 6 fold increase at 3 and 6 h, which falls progressively to a 2 fold increase at 24 h. The maximum transcriptional induction observed with IL-6 is therefore separated in time from the peaks of transcriptional activation seen with IL-1ß or TNFα by at least 6 h suggesting that the kinetics of promoter induction elicited by IL-1ß and TNFα are quite distinct from those elicited by IL-6. To establish the utility of the pGL2-SAA2pt construct in evaluating the activity of inflammatory antagonists the modulatory effect of hIL-1ra in all of the above pro-inflammatory treatment groups was tested. Luciferase reporter readout from cells treated with hIL-1ra alone (Fig.2:B) did not differ significantly from cells treated with medium only (Fig.2:A). When hIL-1ra was added together with IL-6 (Fig.2:F) or TNFα (Fig.2:H) the levels of induction at all timepoints were the same as those seen in the absence of hIL-1ra (Figs.2:E and 2:G respectively). However, when hIL-1ra was added with IL-1ß (Fig2:D) it completely blocked any IL-1ß driven transcriptional activation at all timepoints assayed. These results are in accord with the known specificity of hIL-1ra for the IL-1 receptors (Arend et al., 1989). Thus hIL-1ra effectively and specifically blocks activation of the SAA2 promoter by IL-1ß but not IL-6 or TNFα demonstrating the use of the pGL2-SAA2pt construct in assessing the capacity of known and potential antagonists to down-regulate particular signalling pathways in acute inflammation.

Example 4

Induction of pGL2-SAA2pt by combination inflammatory agonist treatments in the presence or absence of hIL-1ra

The suitability of the assay for studying and isolating components of complex cytokine-driven responses by treating the transiently transfected HepG2 cells both with combinations of the recombinant cytokines and with MoCM (which represents a more physiological complex mixture of pro-inflammatory mediators) was established. Although these

experiments were part of the same assay as the individual cytokine treatments the results are presented separately due to the dramatically different levels of transcriptional induction observed. Figure 3 shows the relative SAA2 promoter activity induced in cells treated with cytokine agonist combinations and/or hIL-1ra. The combined agonist treatments result in levels of transcriptional induction up to ten times greater than those observed for the single cytokine treatments depicted in Figure 2. IL-1ß or TNFα and IL-6 in combination (Figs.3:E and 3:H respectively) results in a dramatic synergistic transcriptional induction that is most evident at the two early timepoints and which subsequently decreases in magnitude over time, i.e. for IL-1ß 70, 67, 43 and 37 fold at 3, 6, 12 and 24 hours respectively. For TNFα the induction at the same timepoints was 63, 45, 20 and 13 fold respectively. Synergistic induction of SAA mRNA by IL-1ß and IL-6 has been reported previously in the human hepatoma cell lines PLC/PRF/5 (5.8 fold over IL-1ß alone) (Steel and Whitehead, 1991), Hep 3B (Ganapathi et al., 1988) and Hep G2 (Betts et al., 1993). TNFα and IL-6 have also been reported to synergistically increase A-SAA mRNA levels in the transformed endothelial cell line ECV304 (Steel et al., 1996). Although attaining levels 10 fold higher, the kinetics of transcriptional induction achieved by IL-1ß (or TNFα) and IL-6 in combination is qualitatively similar to that achieved by IL-6 alone (i.e. both are greater at early timepoints). At 3 h IL-6 alone (Fig.2:E) stimulates the SAA2 promoter to levels similar to those reached by IL-1ß (Fig.2:C) and TNFα (Fig.2:G). Taken together the above results indicate that IL-6 is integral to the early massive synergy observed with IL-1ß and TNFα (Fig.3:E and 3:H). Treatment of cells with MoCM, which contains a complex mixture of inflammatory mediators, may provide a more authentic stimulus (i.e., be closer to that acting in vivo) than treatment with recombinant cytokines either singly or in combination. In previously reported titration experiments involving a number of cell lines (Steel et al., 1996) maximum A-SAA mRNA levels at 24 h were achieved using a 1/20 dilution of MoCM. Treatment of pGL2-SAA2pt transfected HepG2 hepatoma cells with this concentration of MoCM results in an approximately 62 fold increase in transcription at 3 h (Fig.3:C). This transcriptional increase is maintained through 6 h but falls to 30 fold at 12 and 24 h. The induction pattern observed is similar to that seen when cells are treated with a combination of IL-6 and either IL-1ß or TNFα. Human IL-1ra prevented the massive synergy caused by the simultaneous application of IL-1ß and IL-6 (Fig.3:F) bringing the levels of transcriptional induction down to those seen with IL-6 alone (Fig.3:G). Abolition by hIL-1ra of the IL-1ß but not the IL-6 driven component of the synergistic transcriptional activation of the SAA2 promoter demonstrates that the hIL-1ra mediated inhibition of reporter readout is via a specific antagonistic effect rather than a toxic effect. Inclusion of hIL-1ra with TNFα and IL-6 (Fig.3:I) did not affect luciferase reporter readout when compared to the dual addition of these two cytokines

(Fig.3:H). When added with MoCM, hIL-1ra reduced the induction by approximately 20% at 3, 6 and 12h, however by 24 h it caused a 70% reduction in activity. Based on this observation, it is likely that at the early timepoints the synergy effected by cytokines that act early in this system (e.g. IL-6) can be generated in combination with cytokines such as IL-1ß and TNFα to produce maximal induction. As the relative induction capacity of IL-6 becomes less at the later timepoints the contribution of IL-1ß and TNFα, which are themselves becoming more effective when administered singly, may become individually more important in producing a synergy in which antagonism of either IL-1ß or TNFα becomes proportionally (and measurably) significant. Many of the steps involved in cytokine signalling remain ill-defined. IL-1, TNFα and IL-6 each have separate signalling receptors on hepatoma cells: IL-1R1, TNFαR-I and IL-6R respectively. Although some of the membrane proximal signal transduction events consequent to IL-1ß and TNFα binding to their respective receptors are probably distinct, the membrane distal events appear to involve a shared pathway(s) leading to activation of the transcription factor NF-kB. In contrast, IL-6 mediated signal transduction differs wholly from that mediated by IL-1ß and TNFα. The assay reported here will allow distinctions between different inflammatory cytokine and antagonist effects to be made at several levels: (i) extra-cellular neutralizing agents that interact with individual cytokines may be identified; (ii) antagonists specific for particular receptors may be distinguished; (iii) agents that differentially interfere with membrane proximal events may be characterized; and (iv) agents that selectively interfere with membrane distal events may be characterized. In all of the above, the non-affected signalling pathway(s) gives a positive readout suggesting that the effect observed is a specific biological one and not merely the result of toxicity of the agent under test.The construct pGL2-SAA2pt containing the SAA2 promoter is rapidly and consistently transfected into liver cells and is effectively stimulated by the inflammatory mediators MoCM, IL-1ß, TNFa and IL-6 in a manner that closely mimics the response of the endogenous SAA2 gene to these mediators in established hepatoma culture systems. Therefore, an in vitro acute phase response in hepatoma cells may be generated with these cytokines and their differential and combinatorial pro-inflammatory capacity may be accurately detected and measured in the rapid assay based on the luciferase reporter readout driven by the sensitive SAA2 promoter. Timecourse experiments demonstrate that the kinetics of induction of the SAA2 promoter by IL-1ß and TNFa differs significantly from that observed with IL-6. Consequently, future investigations into the induction of acute phase genes should take into account variations in the kinetics of different components of acute phase stimuli. Furthermore the IL-1 receptor antagonist, hIL-1ra, either completely blocks or leaves unchanged the cytokine driven transcriptional induction of the SAA2 promoter in a manner that is entirely dependent on the

presence or absence respectively of IL-1ß from the underlying pro-inflammatory stimulus. The pGL2-SAA2pt construct will be useful both for the basic investigation of the kinetics of inflammatory signalling in a cytokine specific manner, and for evaluating the effect of novel natural and synthetic molecules with potential pro- and anti-inflammatory activities.

Example 5

a) SAA2 5'-UTR/luciferase constructs

The SAA2 5'-UTR (SAA enhancer) was investigated using the luciferase reporter constructs shown in Fig. 4.1. The SAA2 5'-UTR was cloned in both the sense and antisense orientations between the SAA2 promoter and the luciferase coding region.

The mRNA transcribed from the antisense construct (Anti 5'/Luc 3', Fig. 4.1ii) has a 5'-UTR with the nucleotide sequence of the complementary strand of the SAA2 5'-UTR. The antisense SAA2 5'-UTR has no native equivalent and is therefore unlikely to contain any physiologically relevant elements that can influence the luciferase readout. Therefore this construct was used as a control for the study of the sense SAA2 5'-UTR construct (Sense 5'/Luc 3', Fig. 4.1i).

The native SAA2 mRNA and the Sense 5'/Luc 3' construct both have the Kozak consensus sequence, 5'-**ACCAUGG**-3' (SEQ ID NO:5), therefore a second antisense control construct was needed in order to control for the contribution of the Kozak consensus per se to the translational efficiency of the sense construct (Anti Koz 5'/Luc 3', Fig. 4.1iii).

A construct, Luc 5'/Luc 3', containing no SAA2 5'-UTR-derived sequences within its 5'-UTR was also included in the initial experiment. This construct contains 743 bp of the SAA2 promoter and a 42 bp 5'-UTR derived from luciferase 5'-UTR (Fig. 4.1iv). Deletion analyses of the SAA2 promoter region indicated that there are no transcriptional elements more than 400 bp upstream of the SAA2 5'-UTR that have a significant effect on the activation of the SAA2 promoter in HepG2 cells in response to IL-1ß and IL-6 either individually or in combination. Therefore, although this construct contains an addtional 43 bp of SAA2 upstream region compared to the other three constructs, transcription is driven by a functionally identical promoter.

b) Transient transfections

The method of transfection chosen was electroporation which was first described in 1982 (Neumann, E., et al., EMBO J. 1 (1982) 841-845; Wong, T.K., and Neumann, E., Biochem. Biophys. Res. Commun. 107 (1982) 584-587). When mammalian cells are exposed to a brief high voltage electric field, ion channels in the cell membranes open transiently to allow uptake of plasmid DNA. Electroporation is more reproducible than chemical methods such as calcium phosphate precipitation, because exactly the same parameters can be used each time. The main disadvantage of electroporation is the extent of cell death; maximum transfection efficiency occurs when the electrical pulse causes 40-80% cell death. In practice this means that large numbers of cells are needed, particularly if the transfected cells are to be harvested for RNA extraction.

Transient transfection of plasmids was preferred to the generation of stably transfected cell lines. Stable transfectants are grown from single colonies that have to be passaged through many generations before there are sufficient numbers for analysis and increasing passage number may cause phenotypic change. It was therefore considered that transient transfections performed on early passage number cells grown in bulk would provide better quantitative and qualitative consistency.

The cell line chosen was the human hepatoma cell line HepG2. The primary site of production of A-SAA is the liver and HepG2 cells have been shown to produce A-SAA mRNA in response to a range of inflammatory mediators such as IL-1ß and IL-6 (Steel, D.M., et al., Scand. J. Immunol. (1996), "Expression and regulation of constitutive and acute phase serum amyolid mRNAs in hepatic and non-hepatic cell lines", in press. HepG2 cells transiently transfected with SAA2 promoter driven luciferase constructs had previously been used to characterize SAA2 promoter activity (Uhlar, C.M., J. Immunol. Methods 203 (1997) 123-130).

Cells were co-transfected with a second reporter plasmid to control for slight variations in electroporation efficiencies caused by various factors, e.g., differences in plasmid purity and delivery of slightly different pulses to the cells due to small changes in the output from the power pack, and to control for variations in the aliquotting of cells from the same electroporated sample. A plasmid for efficiency control was used and the Rous sarcoma virus (RSV) promoter linked to the ß-galactosidase reporter gene. The RSV promoter is unresponsive to the inflammatory mediators (IL-1ß and IL-6) used.

The ß-galactosidase assay is sensitive, relatively easy to perform and rapid (up to 1 hour), and like the luciferase assay does not require radioactivity. Both the luciferase and the ß-galactosidase assays can be carried out on lysates of transfected cells prepared using Reporter Lysis Buffer (Promega). Thus each cell lysate (approximately 500 µl) can be assayed for both luciferase activity (which requires 100 µl) and ß-galactosidase activity (which requires 150 µl). Therefore the ß-galactosidase measurement can be used as an internal control to correct the luciferase readout for discrepancies between and within transfections.

c) Experimental design

In general each construct was treated as follows:

(1) The Luciferase reporter construct and the ß-galactosidase control plasmid were transfected into HepG2 cells by electroporation.
(2) Transfected cells were aliquotted into the required number of 6-well plates (the number depending on the number of timepoints).
(3) Cells were incubated for 20 hours.
(4) Medium was removed from the cells and replaced with fresh medium (1 ml/well) containing IL-1ß + IL-6, IL-1ß alone or IL-6 alone (each cytokine at a concentration of 10 ng/ml).*
(5) Cells were harvested at intervals between 0 and 48 hours.
(6) Luciferase and ß-galactosidase activities werer assayed immediately after harvesting.

* The zero hour timepoint cells were harvested without being treated with fresh medium.

The ratio of luciferase readout to ß-galactosidase readout was calculated for each lysate. The average value for identically treated samples was calculated; each treatment at each timepoint was performed in triplicate for each construct. These values were then plotted.

Constructs that were to be directly compared were transfected at the same time into aliquots of cells from the same batch of HepG2 cells. The cells from each electroporation were treated identically and assayed for luciferase and ß-galactosidase activities at the same times. In this way the ratios of luciferase to ß-galactosidase activities (Luc/ß-gal) could be directly compared between transfections of different constructs. Separate transfections were carried out for each treatment: IL-1ß + IL-6, IL-6 alone, or IL-1ß alone (Example 5d).

d) Transfections of the SAA2 5'-UTR/luciferase constructs

(I) Treatment with IL-1ß + IL-6

Four T180 flasks of HepG2 cells were grown to 85-90% confluence, the cells pooled and four aliquots prepared for electroporation with the four constructs shown in Fig. 4.1. Each transfection was diluted into 19 ml of complete medium and aliquotted (1 ml/well) onto three wells of six 6-well plates as shown in Fig. 4.2. The following day the transfected cells (except those to be harvested at zero hours) were treated with IL-1ß + IL-6 (both cytokines at 10 ng/ml). The cells were harvestedat 0, 1.5, 3, 6, 12 and 24 hours and assayed for luciferase and ß-galactosidase. The ratio of Luc/ß-gal was calculated for each cell lysate and the average value for each construct at each timepoint calculated (each timepoint was performed in triplicate). The results are plotted in Fig. 4.3.

(II) Treatment with IL-6 alone

Three T180 flasks of HepG2 cells were grown to 85-90% confluence, the cells pooled and three aliquots electroporated: one with the Sense 5'/Luc 3' construct, one with the Anti 5'/Luc 3' construct and the third with the Anti Koz 5'/Luc 3' construct (Fig. 4.1 i, ii and iii). Each transfection was diluted into 22 ml of complete medium and aliquotted (1 ml/well) onto three wells of each of seven 6-well plates in the same way as before (one row of wells was left empty). The following day the cells were treated with 10 ng/ml IL-6, except those to be harvested at zero hours. The cells were harvested at 0, 1.5, 3, 6, 12, 24 and 48 hours, the luciferase and ß-galactosidase activities measured and the average Luc/ß-gal values for each construct at each timepoint calculated. The results are plotted in Fig. 4.4.

(III) Treatment with IL-1ß alone

Three flasks of HepG2 cells were prepared and transfected with the Sense 5'/Luc 3', Anti 5'/Luc 3' and Anti Koz 5'/Luc 3' constructs as above. The transfected cells were diluted into 22 ml of complete medium and aliquotted (1 ml/well) onto three wells of each of seven 6-well plates. The following day the cells were treated with 10 ng/ml IL-1ß, except those to be harvested at zero hours. The cells were harvested at 0, 3, 6, 12, 24, 36 and 48 hours and the luciferase and ß-galactosidase activities measured. The average Luc/ß-gal value for each construct at each timepoint was calculated and the results plotted in Fig. 4.5.

Example 6

Luciferase mRNA analysis

a) Quantitative S1 nuclease protection assay

Full interpretation of the results in Example 5 requires quantitation of the levels of luciferase mRNA transcribed from the Sense 5'/Luc 3' and Anti Koz 5'/Luc 3' constructs after cytokine treatment, to permit the efficiency of translation per unit of each chimeric mRNA to be assessed. Luciferase mRNA levels were measured by quantitative S1 nuclease protection. This procedure utilizes the activity of S1 nuclease wich hydrolyzes single-stranded but not double-stranded nucleic acids, i.e., single-stranded DNA hybridized to mRNA is protected from S1 nuclease digestion whereas unhybridized single-stranded DNA is digested. The principles of the procedure are outlined in Fig. 4.6.

The quantitative S1 nuclease protection assay uses oligonucleotides that are complementary to at least 40 nucleotides of a target mRNA. The oligonucleotides are designed to be complementary at their 5'-ends, where they are labelled with [γ-^{32}-P]-ATP, but mismatched for at least 10 nucleotides at their 3'-ends. Once hybridized to the target mRNA only the mismatched 3'-end can be hydrolyzed by S1 nuclease. The labelled protected fragment can be quantitatively analyzed on a denaturing polyacrylamide gel.

Two 75-mer oligonucleotides were designed to permit the levels of luciferase mRNA in the transfected cells to be measured. The Luc S1 oligo was designed such that the first 60 nucleotides were complementary to part of the coding region of the luciferase reporter gene mRNA; the final 15 nucleotides were mismatched so that purines were opposite purines and pyrimidines were opposite pyrimidines (Fig. 4.7a; SEQ ID NO:6). The GAPDH S1 oligo was designed such that the first 45 nucleotides were complementary to part of the coding region of the mRNA of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene; the remaining 30 nucleotides were mismatched as above (Fig. 4.7b; SEQ ID NO:7). GAPDH is a house-keeping gene, the mRNA levels of which are unaffected by cytokine treatment in HepG2 cells. This message is therefore an ideal internal control against which changes in luciferase mRNA levels can be measured. This ß-galactosidase mRNA present in the RNA samples from the transfected cells could not be detected by this technique and so could not be used as an internal control.

b) Experimental design

Quantitative S1 nuclease protection was used to measure the mRNA levels transcribed from the Sense 5'/Luc 3' and Anti Koz 5'/Luc 3' constructs after treatment with IL-1ß and IL-6. The luciferase and ß-galactosidase readouts as well as the mRNA levels had to be measured at each timepoint to ensure that the Luc/ß-gal values followed the same pattern as that observed in Fig. 4.3. The procedure was as follows:

(1) Four 85-90% confluent T180 flasks were grown up, combined and prepared for transfection.
(2) Each construct was electroporated into two separate aliquots of cells*.
(3) Cells transfected with the same construct were pooled.
(4) The transfected cells were diluted into 37 ml of complete medium.
(5) 24 ml of the cells were aliquotted onto six 20 mm tissue culture plates (4 ml/plate)**.
(6) 12 ml of the cells were aliquotted 1 ml/well onto two wells of each of six 6-well plates**.
(7) The cells were incubated for 20 hours.
(8) The medium was removed and replaced with fresh medium containing 10 ng/ml IL-1ß + 10 ng/ml IL-6 (4 ml per 20 mm plate and 1 ml per well of the 6-well plates).
(9) The 20 mm plates were harvested for RNA and the 6-well plates for luciferase and ß-galactosidase at 0, 1.5, 3, 6, 12 and 24 hours.

* Each construct was transfected into two aliquots rather than one, in order to increase the numbers of cells on each 20 mm plate and therefore increase the yields of RNA.

** The ratio of the surface areas of the 20 mm plates to that of the wells of the 6-well plates is 4:1. Aliquotting 4 ml of cells onto the 20 mm plates and 1 ml onto the wells of the 6-well plates therefore ensured that the cells were seeded at equal densities and were therefore at similar levels of confluence when treated with cytokines.

The RNA harvested at each timepoint for each construct was treated with RQ1 DNase, as contaminating plasmid DNA would hybridize to the probe and be protected. 25 µm of the RQ1 DNase treated RNA sample was hybridized to ~10^5 cpm of end-labelled oligo probe mix and the S1 nculease protection assay carried out. The protected fragments were resolved on a 6% denaturing polyacrylamide gel, the gels dried and exposed to film. Fig. 4.8 shows an autoradiograph of one such set of reactions. The total amount of RNA that could be extracted at each timepoint was limited to between 60 and 70 µg due to the realtively small numbers of

cells on each 20 mm plate at each timepoint. Treatment of the crude RNA preps with RQ1 DNase, followed by further purification reduced this yield to between 30 and 40 µg. As each S1 reaction required 25 µg of purified RNA, only one such reaction could be carried out per timepoint (Fig. 4.8).

Each mRNA gives rise to more than one band as evident from Fig. 4.8. This is due to a small degree of overdigestion by S1 nuclease at the 3'-end of the protected portion of the probe and is similar to the results observed in other experiments. This overdigestion at the 3'-end has no impact on the quantitative aspect of the analysis, as the probe is labelled at the 5'-end and therefore the amount of radiolabelled protected probe is unaffected.

The intensitities of the luciferase and GAPDH mRNa bands were measured using an INSTANT IMAGER (Packard Instrument Company, Meriden, CT, USA). The ratio of the intensity of the luciferase mRNA bands to that of the GAPDH mRNA bands was calculated for each construct at each timepoint. These values measure the levels of chimeric luciferase mRNA produced by each construct and are plotted in Fig. 4.9a. This is a representative result of S1 reactions carried out on RNA extracted from cells transfected with these two constructs. Errors could not be calculated as each reaction could only be carried out once.

The luciferase and ß-galactosidase activities were measured, the average Luc/ß-gal value calculated for each timepoint calculated, and the values plotted (Fig. 4.9b).

c) Primer extension analysis of SAA2 5'-UTR/luciferase constructs

Primer extension was used to check that transcription started at the same point for both the sense and the antisense constructs. An oligo (PrEx1) was designed to be complementary to 30 nucleotides at the 5'-end of the luciferase coding region. This oligo was end-labelled with [γ-^{32}P]-ATP and hybridized to RNA harvested from cells transfected with the Sense 5'/Luc 3' and Anti Koz 5'/Luc 3' constructs (the RNA was treated with RQ1 DNase before hybridization). The primer was then extended using cold deoxynucleotides to map the 5'-ends of the two transcripts.

The products of the primer extension reactions were run out on a 6% polyacrylamide gel alongside sequencing reactions carried out with the unlabelled PrEx1 oligo on the Sense 5'/Luc 3' and Anti Koz 5'/Luc 3' plasmids. The products of both reactions are the same size, indicating that the 5'-UTRs of both constructs are the same length. Comparison of the primer

extension reactions with the sequencing reactions shows that the 5'-ends of both chimeric transcripts map to the predicted starts of the 5'-UTRs.

List of References

Arend, W.P., et al., J. Immunol. 143 (1989) 1851

Baumann, H., and Gauldie, J., Immunol. Today 15 (1994) 74

Baumann, H., and Gauldie, J., Mol. Biol. Med. 7 (1990) 147

Beach, C.M., et al., Biochem. J. 282 (1992) 615

Betts, J.C., et al., J. Biol. Chem. 268 (1993) 25624

Betts, J.C., et al., Scand. J. Immunol. 34 (1991) 471-482

Dwulet, F.E., et al., Biochemistry 27 (1988) 1677

Felgner et al., Proc. Natl. Acad. Sci. USA 84 (1987) 7413

Fey, G.H., and Gauldie, J., In: H. Popper and F. Schaffner (Eds.), Progress in Liver Diseases. Vol.9. (1989) WB Saunders, Philadelphia, p.89

Friedman, T., Science 244 (1989) 1275

Ganapathi, M.K., et al., Biochem. Biophys. Res. Commun. 157 (1988) 271

Hoffman, J.S., and Benditt, E.P., J. Biol. Chem. 257 (1982) 10510

Kushner, I., and Mackiewicz, A., Disease Markers 5 (1987) 1

Kushner, I., Ann. NY Acad. Sci. USA 389 (1982) 39

Kushner, I., et al., Ann. N.Y. Acad. Sci. 389 (1982) 235

Margolskee et al., Mol. Cell. Biol. 8 (1988) 2937

Marhaug, G., Scand. J. Immunol. 18 (1983) 329

McLughlin, J. Virol. 92 (1988) 1963

Morgan 1993, RAC Data Management Report, June 1993

Moss et al., Ann. Rev. Immunol. 5 (1987) 305

Mullen, C.A., Proc. Natl. Acad. Sci. USA 89 (1992) 33-37

Mulligan, R.C., (1991) in Nobel Symposium 8: Etiology of human disease at the DNA level (Lindsten, J., and Pattersun, eds.) 143-189, Raven Press

Neumann, E., et al., EMBO J. 1 (1982) 841-845

Rasmussen et al., Methods Enzymol. 139 (1986) 642

Sambrook et al., "Expression of cloned genes in E.coli" in Molecular cloning: a laboratory manual (1989), Cold Spring Harbor Laboratory Press, New York, USA, 9.47-9.63 and 11.45-11.61

Sipe, J.D., et al., Biochemistry 24 (1985) 2931

Steel, D.M., and Whitehead, A.S., Biochem. J. 277 (1991) 477

Steel, D.M., and Whitehead, A.S., Immunol. Today 15 (1994) 81

Steel, D.M., et al., Genomics 16 (1993) 447-454

Steel, D.M., et al., Scand. J. Immunol. (1996), "Expression and regulation of constitutive and acute phase serum amyolid mRNAs in hepatic and non-hepatic cell lines", in press

Uhlar, C.M., J. Immunol. Methods 203 (1997) 123-130

Wong, T.K., and Neumann, E., Biochem. Biophys. Res. Commun. 107 (1982) 584-587

Woo, P., et al., J. Biol. Chem. 262 (1987) 15790-15795

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: BOEHRINGER MANNHEIM GMBH
(B) STREET: Sandhofer Str. 116
(C) CITY: Mannheim
(E) COUNTRY: Germany
(F) POSTAL CODE (ZIP): D-68305
(G) TELEPHONE: 08856/60-3446
(H) TELEFAX: 08856/60-3451

(ii) TITLE OF INVENTION: Use of an acute phase serum amyloid A gene promoter (A-SAA promoter) in the treatment of diseases in mammals

(iii) NUMBER OF SEQUENCES: 7

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30B (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1218 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TGGCCATCCT TTACTTCCTG GCCCTACCCA TTTCTCAGAG ATTAAAAAAA ATTTTTTAAG 60

ATAATCTTGC TGTGTTGCCC AGGCTGGCCT CAAATTTCTG GGTTCAAGCA GGCCTCCTGC 120

CTTGGCCTCC CAAGTAGCTG GGACTATAGG CACATGCCAC CATGCCTGGC CCATTTCTAA 180

ATTGCTTGTT TGTTTGTTAT TACAAATGCC TAGCCCTCAG GGTATGAACA TGGACTGGAG 240

AAGAAGAAAC CAGAGTTGCT GCTATGTCCA CCAGCCTCTC TGCATGTCCT GGCCTCAGCC 300

CCCCTGGGCT CTGGTACTGA CCCATCTCTG GCCACCATGC TCCTCCATAA GCCTCTGCAG 360

AGCTAATCTG ACCCTGTTGA TGTTCTCATG AGAGAGTGAT CTGCATGCCC CCTGCACCCC 420

TCCGTGATAA TACAGCAGAC CAAGAGCTCT CCCACCCTTC CCTGCCTGGA TGCTGGGCAC 480

GTCCCCAGCT GGGCTGCCTA TTTAACGCAC CACACTCTCA TTCTCCCAAG GTGGGGCTCC 540

AGGACTAGGC TGGGGCAGCA GAAAGTCCCC CTCTCTACAT TGTCCTTGGC TCAGGAGCCA 600

ACTTAGAAAA AGCATTTCCA AATTGGCTAA GCCAGCGGAG CAGAGATTTT CTGTGCTGAG 660

AAATATCAGG ACATCCAGAG GGGTGGAAGG AGGCTTCCAG GGCACACATG AGATGTGGCA 720

GGGGTAGGCT GTCCGTTTTA AAGCTTAAAG CTTTAGACAT GAACTCACAG GGATTTCAGT 780

CAGGGTCATC TGCCATGTGG CCCAGCAGGG CCCATCCTGA GGAAATGACC GGTATAGTCA 840

GGAGCTGGCT GAAGAGCTGC CCTCACTCCA CACCTTCCAG CAGCCCAGGT GCCGCCATCA 900

CGGGGCTCCC ACTGGCATCT CTGCAGCTGC ACTTCCCCCA ATGCTGAGGA GCAGAGCTGA 960

TCTAGCACCC TGTCCATTGC CAAGGCACAG CAAACCTCTC TTGTTCCCAT AGGTTACACA 1020

ACTGGGATAA ATGACCCGGG ATGAAGAAAC CACCGGCATC CAGGAACTTG TCTTAGACCA 1080

GTTTGTAGGG GAAATGACCT GCAGGGACTT TCCCCAGGGA CCACATCCAG CTTTTCTTCC 1140

CTCCCAAGAG ACCAGCAAGG CTCACTATAA ATAGCAGCCA CCTCTCCCTG GCAGACAGGG 1200

ACCCGCAGCT CAGCTACA 1218

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AAGAATTCAC GCGTCCATGC ATGTTGCGGC CGCTTGGCCA TCCTTTACTT CCT 53

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TTGAATTCCT CGAGCAGGTA CCATACATAT GTAGCTGAGC TGCGGGTCC 49

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AGGGACCCGC AGCTCAGCTA CAGCACAGAT CAGCACC 37

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

ACCAUGG 7

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CCCGGTATCC AGATCCACAA CCTTCGCTTC AAAAAATGGA ACAACTTTAC CGACCGCGCC 60

TAAGGGCGAC GAAAA 75

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GCTTCCCGTT CTCAGCCTTG AAGGTGCCAT GGAATTTGCC ATGGGGTTCC GACGCGGTTC 60

CACGTGCCCA ACGTG 75

Patent Claims

1. Use of a gene therapy agent containing a vector with a gene coding for a therapeutically useful protein for the treatment of a mammalian disease, wherein

   a) an inflammatory response consisting of a local in vivo production of inflammatory mediators like IL-6 and/or IL-1ß is induced at a predetermined locus or systemically by said agent;

   b) the gene coding for said protein is under the control of the human acute phase serum amyloid A gene promoter SAA1 or SAA2, and

   c) the expression of said gene is induced by said inflammatory mediators.

2. Use according to claim 1, wherein the gene therapy vector is applied in a single local bolus dose.

3. A process for the in vitro production of a predetermined recombinant protein in eukaryotic cells by expression of a gene with a nucleic acid sequence encoding said protein under the control of a regulatory sequence which contains the human acute phase promoter of human serum amyloid A gene SAA1 or SAA2, and co-expression of transcription factors in said cells, which activates said promoter, and recovering the protein from the host cell culture.

4. Use of a gene therapy vector for the treatment of an inflammatory disease, wherein

   a) said vector contains a nucleic acid which codes for a cytokine-inhibiting protein, which gene is under the control of a human serum amyloid A promoter SAA1 or SAA2;

   b) said nucleic acid is expressed at the site of said inflammatory disease, and

   c) said protein inhibits the inflammatory disease by inhibiting the action of cytokines on cells.

5. An isolated first nucleic acid molecule which codes for a regulatory sequence for the expression of a second nucleic acid cording for a protein, the expression control of which is regulated by inflammatory mediators and inhibitors, wherein said nucleic acid molecule is selected from the group consisting of

   (i) the expression control region shown in SEQ ID NO:1 or the complementary sequence, or

   (ii) an expression control sequence which hybridizes with SEQ ID NO:1 or the complementary sequence under stringent conditions.

6. An isolated nucleic acid molecule which codes for an enhancer element for the expression of a nucleic acid coding for a protein, said enhancer element being selected from the group consisting of

   (i) the expression control region shown in SEQ ID NO:4 or the complementary sequence, or

   (ii) an expression control sequence which hybridizes with SEQ ID NO:4 or the complementary sequence under stringent conditions.

7. An expression vector containing the isolated nucleic acid molecule of claim 5.

8. An expression vector containing the isolated nucleic acid molecule of claim 6.

9. An expression vector containing the isolated nucleic acid molecules of claims 5 and 6.

Figure 1a

```
TGGCCATCCTTTACTTCCTGGCCCTACCCATTTCTCAGAGATTAAAAAAAATTTTTTAAGATAATCTTGC   -1127
TGTGTTGCCCAGGCTGGCCTCAAATTTCTGGGTTCAAGCAGGCCTCCTGCCTTGGCCTCCCAAGTAGCTG   -1057
GGACTATAGGCACATGCCACCATGCCTGGCCCATTTCTAAATTGCTTGTTTGTTTGTTATTACAAATGCC    -987
TAGCCCTCAGGGTATGAACATGGACTGGAGAAGAAGAAACCAGAGTTGCTGCTATGTCCACCAGCCTCTC    -917
TGCATGTCCTGGCCTCAGCCCCCCTGGGCTCTGGTACTGACCCATCTCTGGCCACCATGCTCCTCCATAA    -847
GCCTCTGCAGAGCTAATCTGACCCTGTTGATGTTCTCATGAGAGAGTGATCTGCATGCCCCCTGCACCCC    -777
TCCGTGATAATACAGCAGACCAAGAGCTCTCCCACCCTTCCCTGCCTGGATGCTGGGCACGTCCCCAGCT    -707
GGGCTGCCTATTTAACGCACCACACTCTCATTCTCCCAAGGTGGGGCTCCAGGACTAGGCTGGGGCAGCA    -637
GAAAGTCCCCCTCTCTACATTGTCCTTGGCTCAGGAGCCAACTTAGAAAAAGCATTTCCAAATTGGCTAA    -567
GCCAGCGGAGCAGAGATTTTCTGTGCTGAGAAATATCAGGACATCCAGAGGGGTGGAAGGAGGCTTCCAG    -497
GGCACACATGAGATGTGGCAGGGGTAGGCTGTCCGTTTTAAAGCTTAAAGCTTTAGACATGAACTCACAG    -427
GGATTTCAGTCAGGGTCATCTGCCATGTGGCCCAGCAGGGCCCATCCTGAGGAAATGACCGGTATAGTCA    -357
GGAGCTGGCTGAAGAGCTGCCCTCACTCCACACCTTCCAGCAGCCCAGGTGCCGCCATCACGGGGCTCCC    -287
ACTGGCATCTCTGCAGCTGCACTTCCCCCAATGCTGAGGAGCAGAGCTGATCTAGCACCCTGTCCATTGC    -217
CAAGGCACAGCAAACCTCTCTTGTTCCCATAGGTTACACAACTGGGATAAATGACCCGGGATGAAGAAAC    -147
CACCGGCATCCAGGAACTTGTCTTAGACCAGTTTGTAGGGGAAATGACCTGCAGGGACTTTCCCCAGGGA     -77
CCACATCCAGCTTTTCTTCCCTCCCAAGAGACCAGCAAGGCTCACTATAAATAGCAGCCACCTCTCCCTG      -7

      +1
GCAGACAGGGACCCGCAGCTCAGCTACA                                              +20
```

Figure 1b

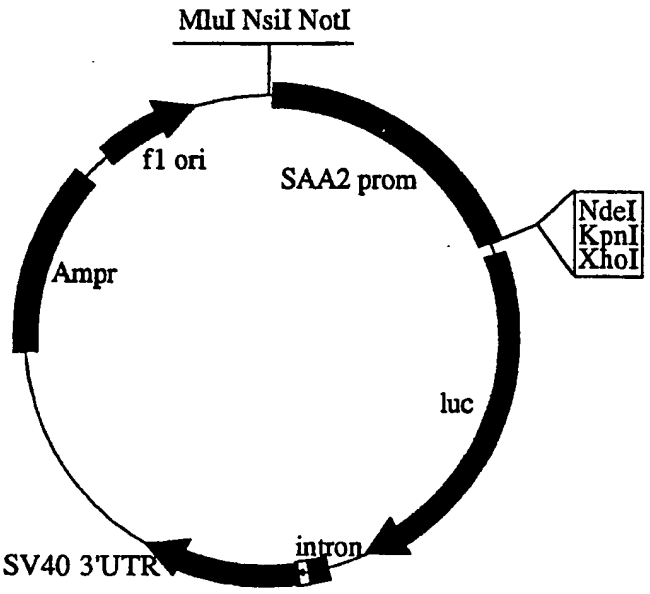


Figure 2

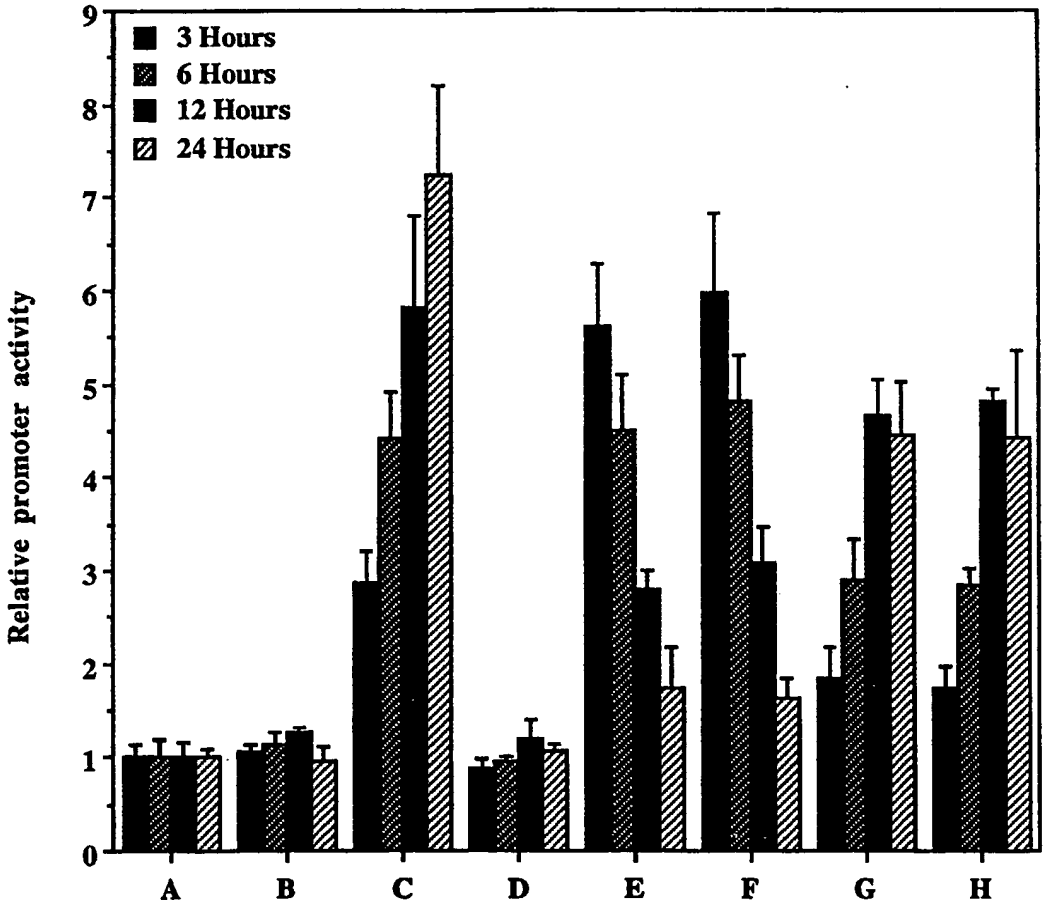

3 Hours
6 Hours
12 Hours
24 Hours
Relative promoter activity
0
1
2
3
4
5
6
7
8
9
A
B
C
D
E
F
G
H

Figure 3

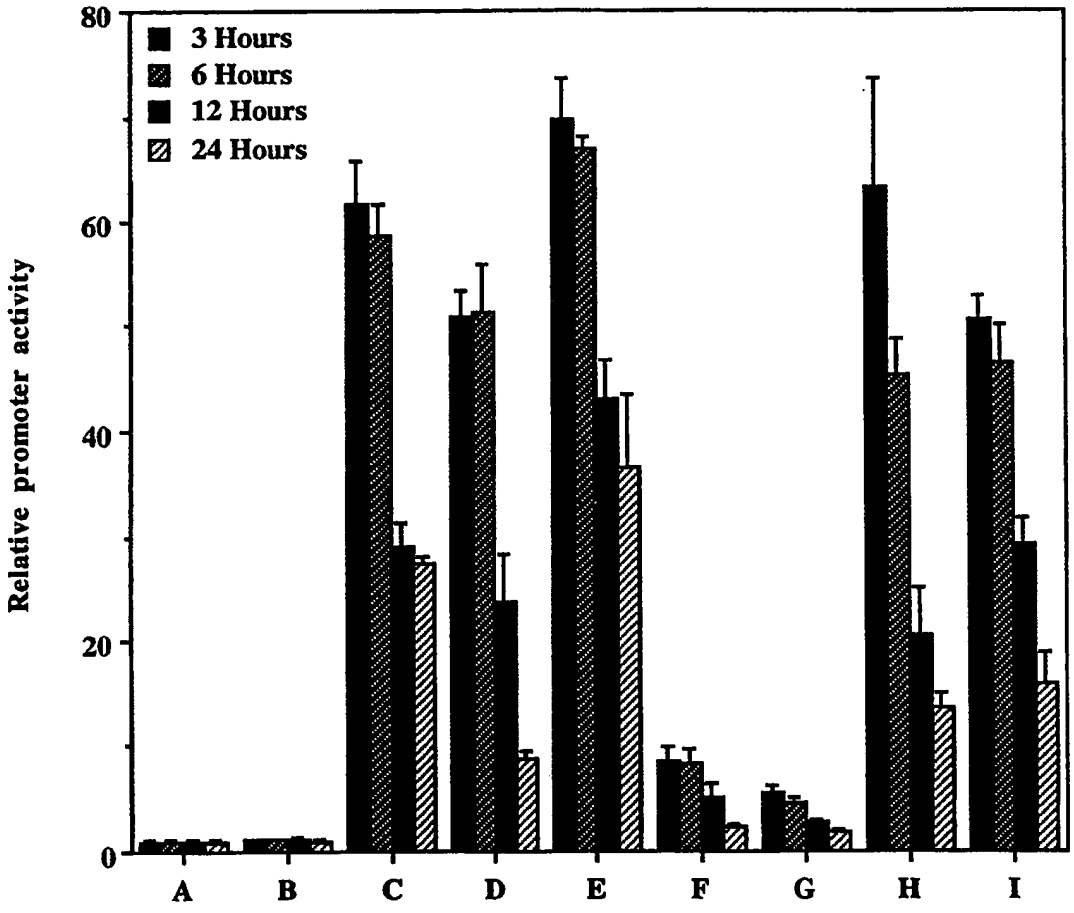

80
60
40
20
0
Relative promoter activity
3 Hours
6 Hours
12 Hours
24 Hours
A
B
C
D
E
F
G
H
I

Figure 4.1

(i) Sense 5'/Luc 3' construct

(ii) Anti 5'/Luc 3' construct

(iii) Anti Koz 5'/Luc 3' construct

(iv) Luc 5'/Luc 3' construct

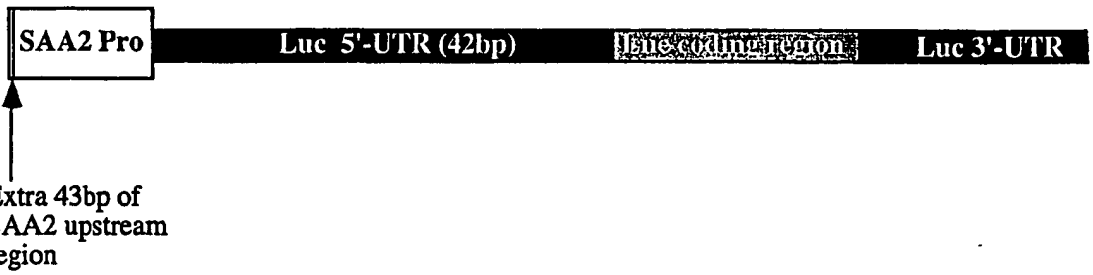
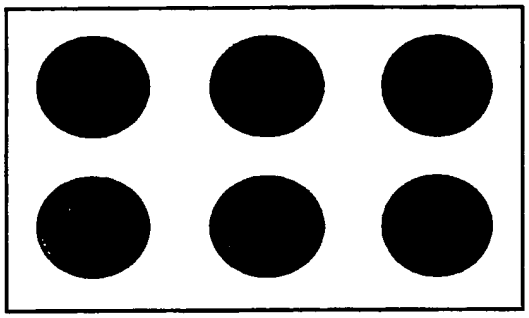
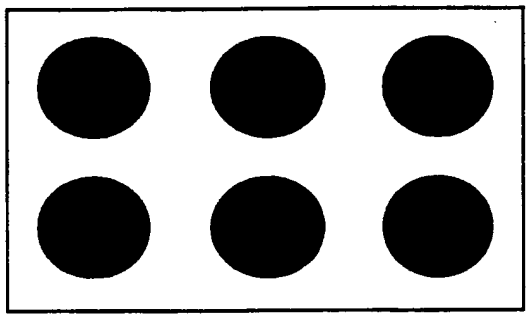


Figure 4.2



= HepG2 cells transfected with Sense 5'/Luc 3' construct

= HepG2 cells transfected with Anti 5'/Luc 3' construct



= HepG2 cells transfected with Anti Koz 5'/Luc 3' construct

= HepG2 cells transfected with Luc 5'/Luc 3' construct

Figure 4.3

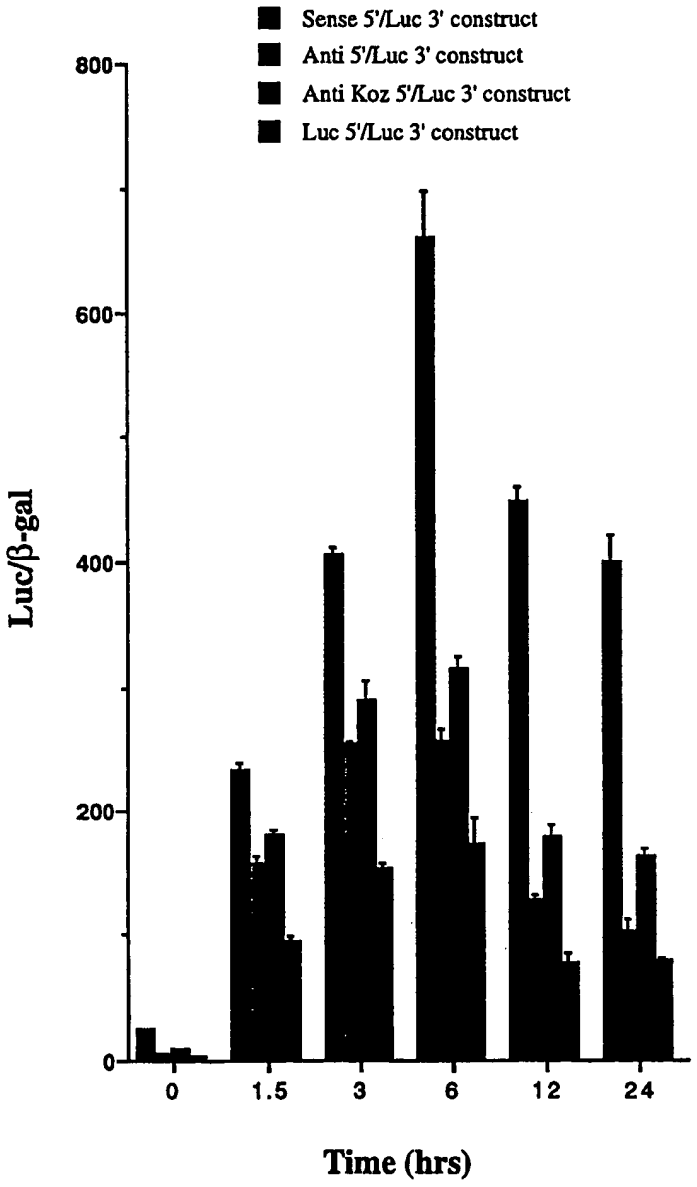

Sense 5'/Luc 3' construct
Anti 5'/Luc 3' construct
Anti Koz 5'/Luc 3' construct
Luc 5'/Luc 3' construct
800
600
400
200
0
Luc/β-gal
0
1.5
3
6
12
24
Time (hrs)

Figure 4.4

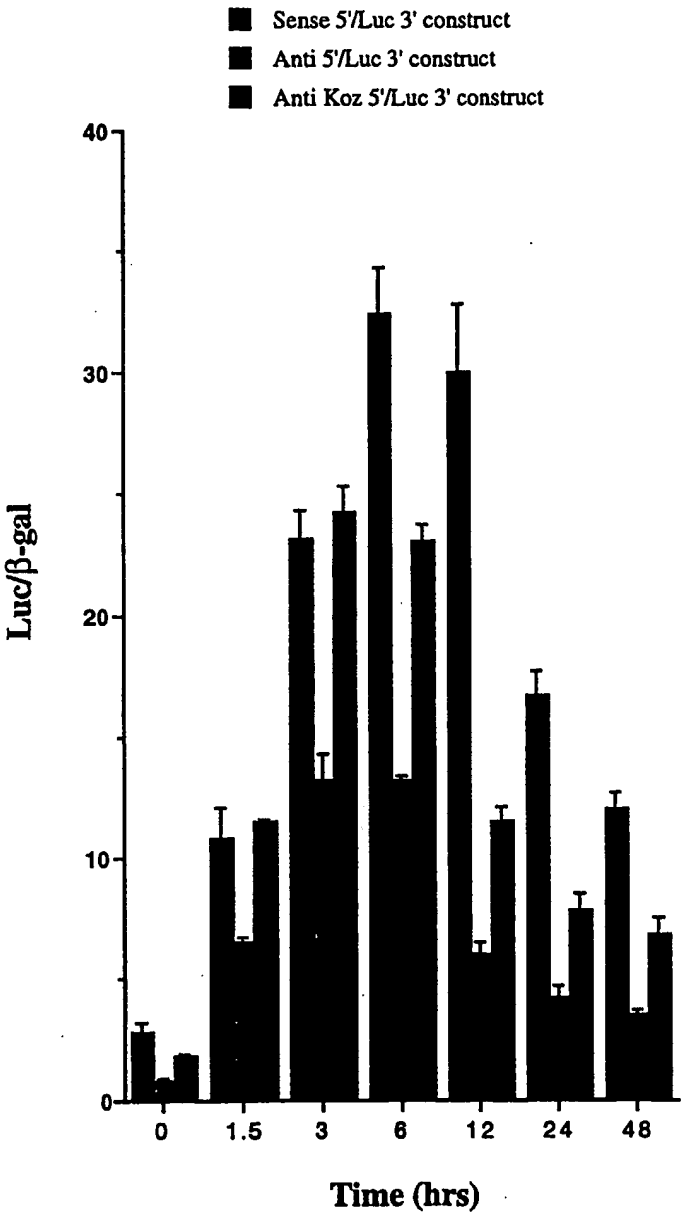

Sense 5'/Luc 3' construct
Anti 5'/Luc 3' construct
Anti Koz 5'/Luc 3' construct
Luc/β-gal
40
30
20
10
0
0
1.5
3
6
12
24
48
Time (hrs)

Figure 4.5

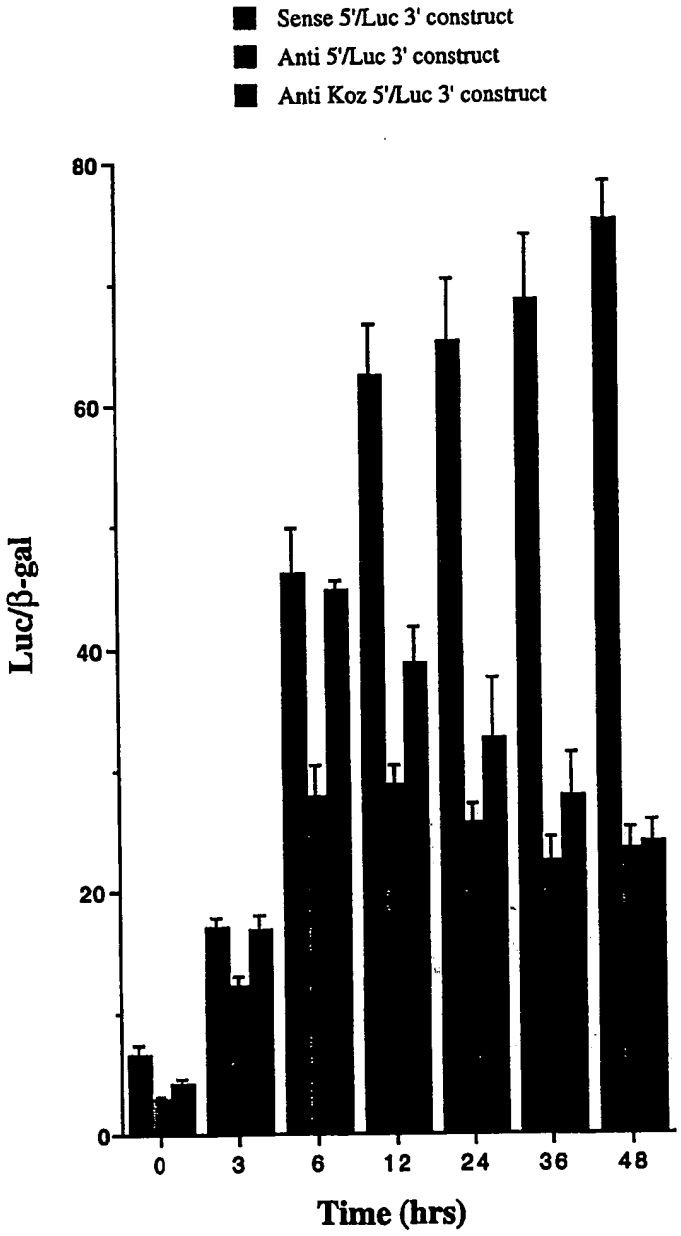

Sense 5'/Luc 3' construct
Anti 5'/Luc 3' construct
Anti Koz 5'/Luc 3' construct
Luc/β-gal
0
20
40
60
80
0
3
6
12
24
36
48
Time (hrs)

Figure 4.6

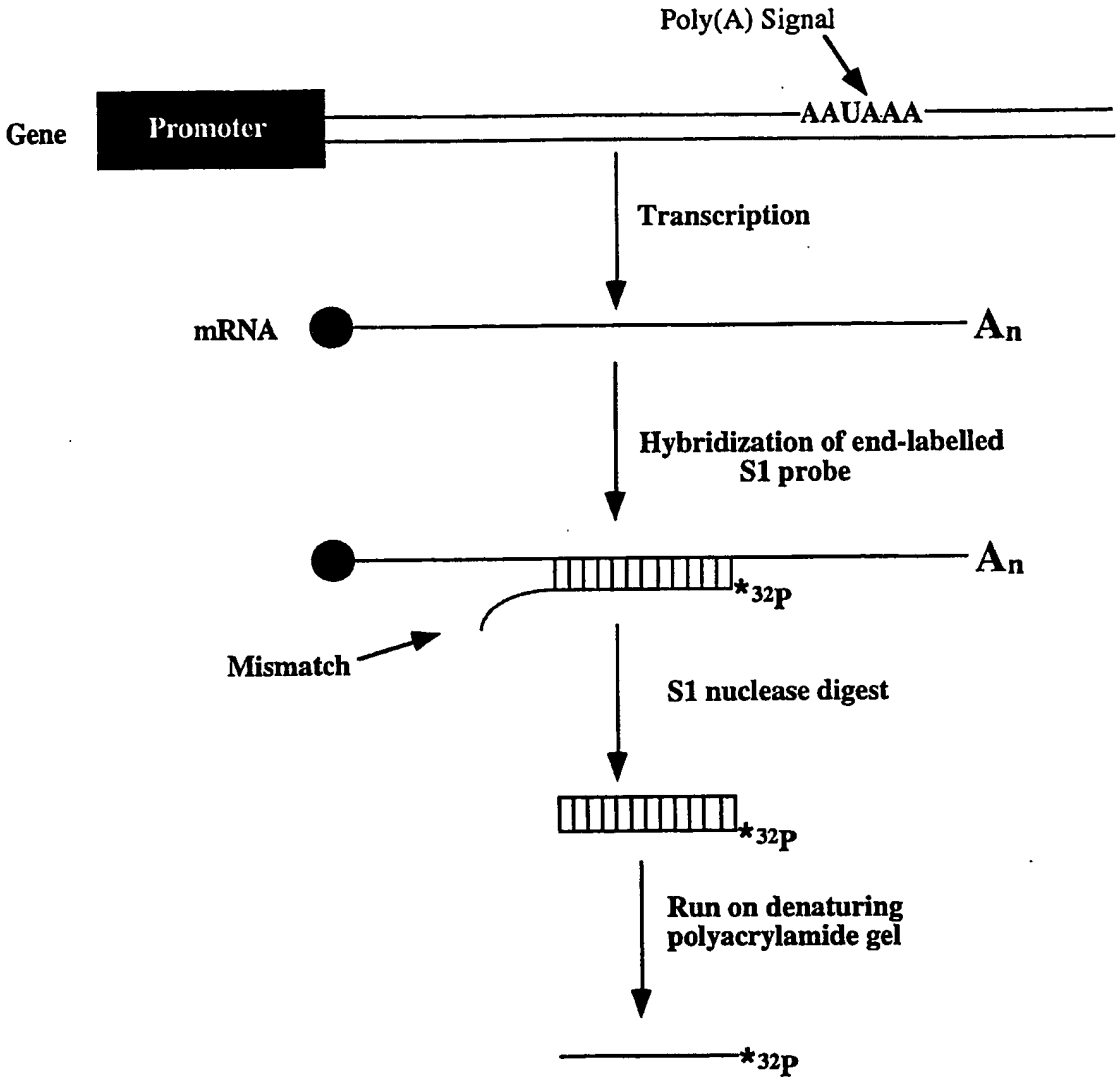

Poly(A) Signal
AAUAAA
Gene
Promoter
Transcription
mRNA
An
Hybridization of end-labelled S1 probe
An
*32P
Mismatch
S1 nuclease digest
*32P
Run on denaturing polyacrylamide gel
*32P

Figure 4.7

(a) Luciferase S1 oligo sequence

5 ´CCCGGTATCCAGATCCACAACCTTCGCTTCAAAAAATGGAACAACTTTACCGACCGCGCCTAAGGGCGACGAAAA

mismatch

(b) GAPDH S1 oligo sequence

5 ´GCTTCCCGTTCTCAGCCTTGAAGGTGCCATGGAATTTGCCATGGGGTTCCGACGCGGTTCCACGTGCCCAACGTG

mismatch

Figure 4.8

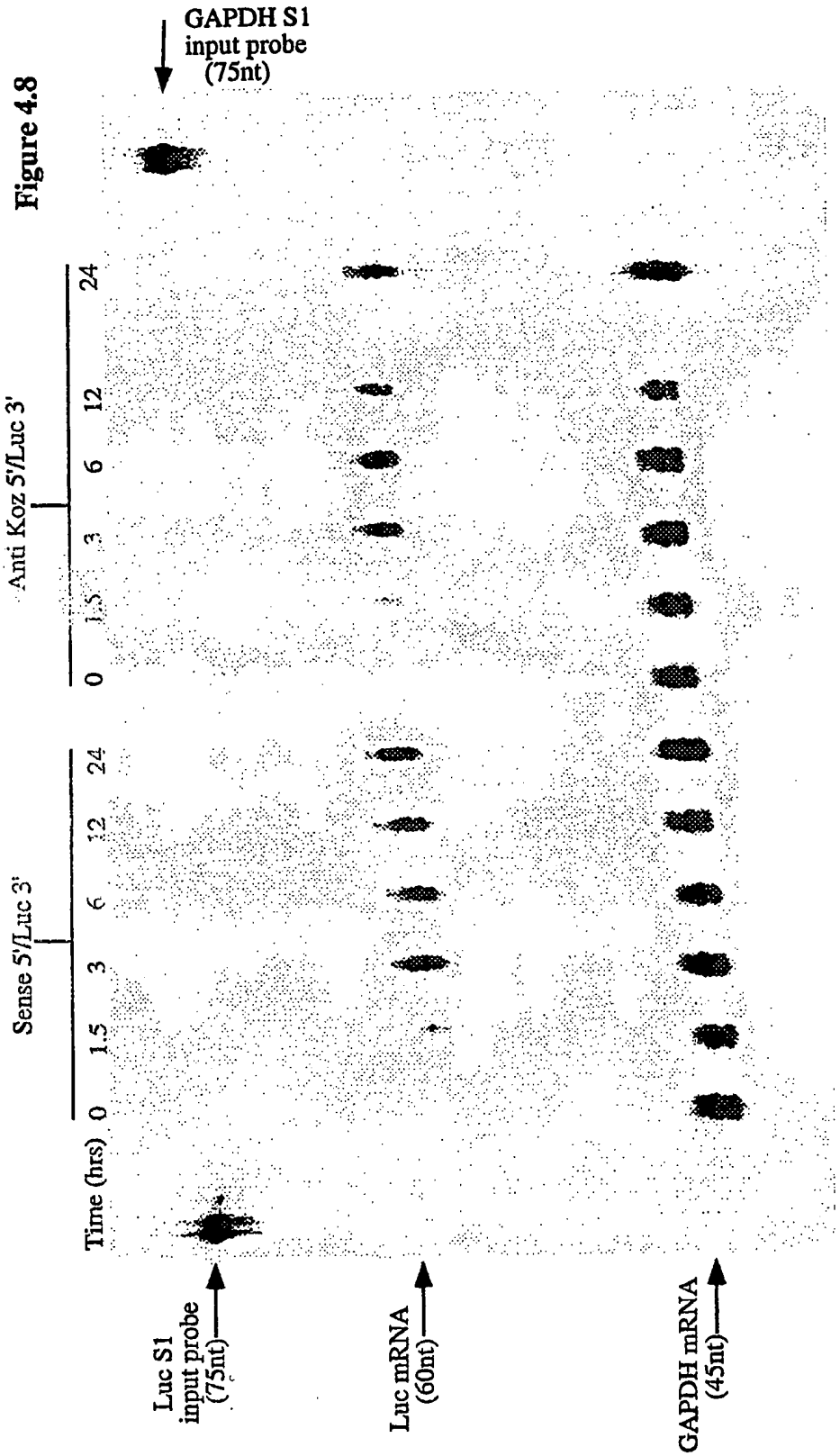

GAPDH S1
input probe
(75nt)
Anti Koz 5'/Luc 3'
Sense 5'/Luc 3'
Time (hrs)
0
1.5
3
6
12
24
Luc S1
input probe
(75nt)
Luc mRNA
(60nt)
GAPDH mRNA
(45nt)

Figure 4.9 a

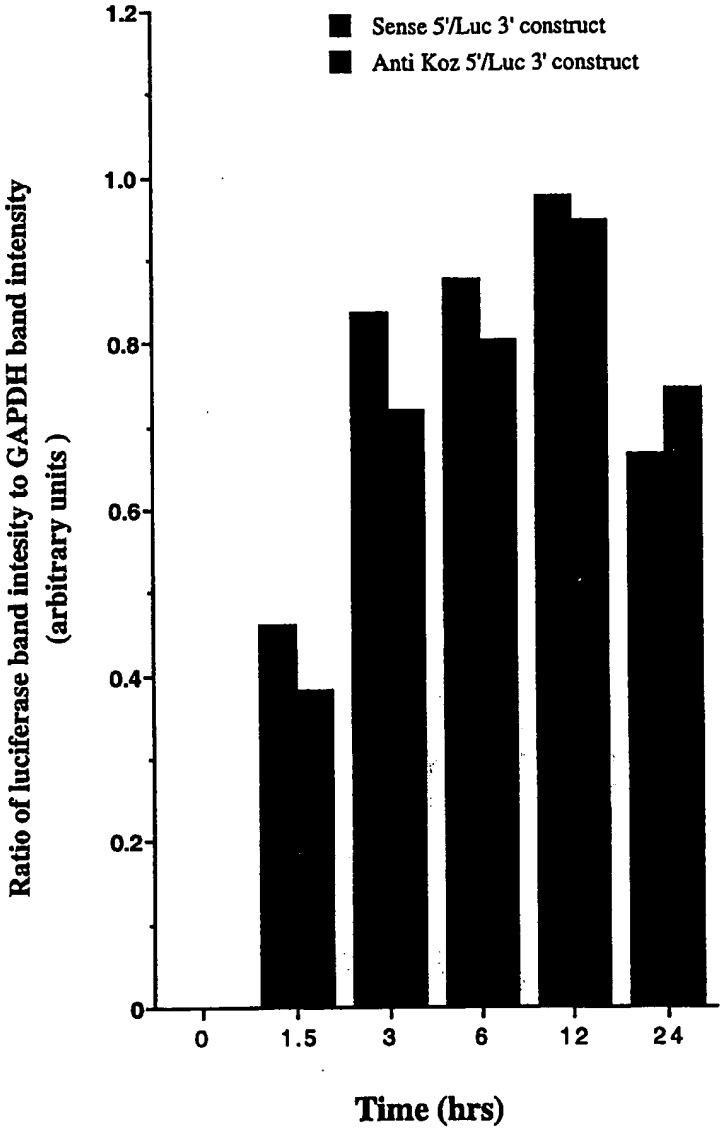
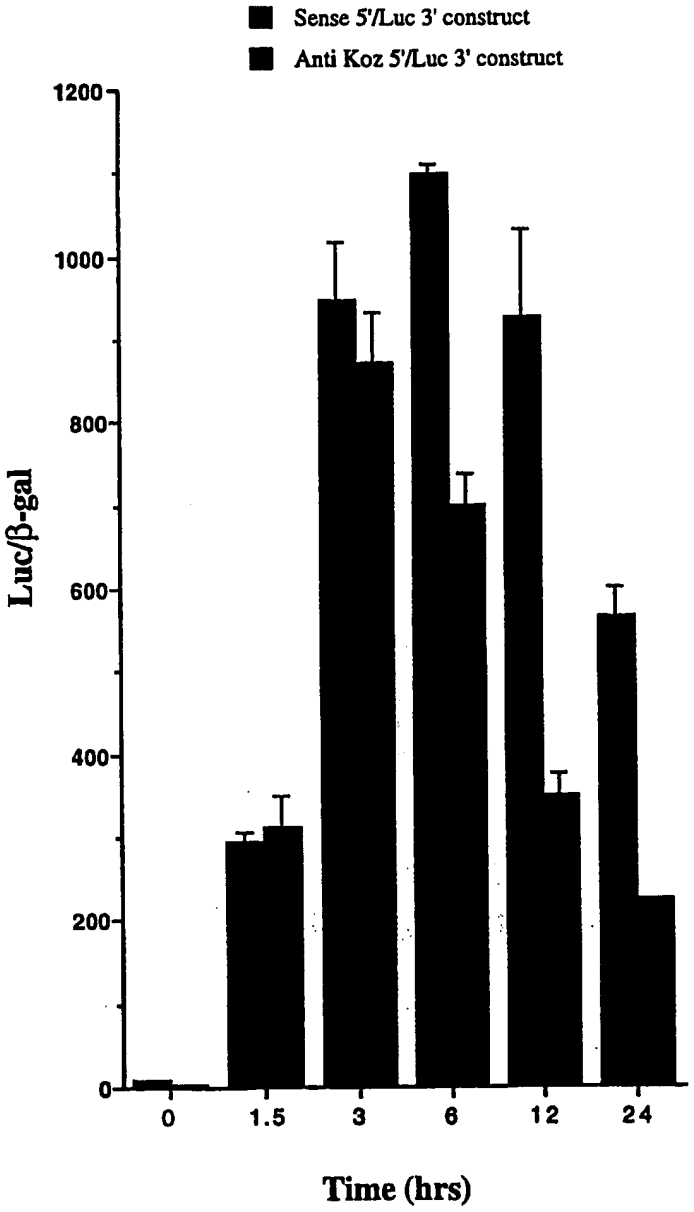

Sense 5'/Luc 3' construct
Anti Koz 5'/Luc 3' construct
Ratio of luciferase band intesity to GAPDH band intensity
(arbitrary units)
1.2
1.0
0.8
0.6
0.4
0.2
0
0
1.5
3
6
12
24
Time (hrs)

Figure 4.9 b


Sense 5'/Luc 3' construct
Anti Koz 5'/Luc 3' construct
Luc/β-gal
1200
1000
800
600
400
200
0
0
1.5
3
6
12
24
Time (hrs)

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/EP 98/01363

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/85 A61K48/00 C12N15/11

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MARK R. EDBROOKE ET AL.: "Constitutive and NF-kappaB-like proteins in the regulation of the serum amyloid A gene by Interleukin 1" CYTOKINE, vol. 3, no. 5, September 1991, pages 380-388, XP002071438 see abstract see page 380, right-hand column, last paragraph - page 381, right-hand column, paragraph 4 see page 384, left-hand column, paragraph 2 - right-hand column, paragraph 1 see page 386, right-hand column, paragraph 2 - paragraph 3 --- -/--	3,5-9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 July 1998	28.07.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Montero Lopez, B

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter ›nal Application No
PCT/EP 98/01363

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	MARK R. EDBROOKE ET AL.: "Identification of cis-acting sequences responsible for phorbol ester induction of human serum amyloid A gene expression via a nuclear factor kappaB-like transcription factor" MOLECULAR AND CELLULAR BIOLOGY, vol. 9, no. 5, May 1989, WASHINGTON US, pages 1908-1916, XP002039444	5-9
A	see abstract; figure 1 see page 1911, right-hand column, paragraph 1 - page 1912, left-hand column, paragraph 3 see page 1912, right-hand column, paragraph 1 - paragraph 4 see page 1912, right-hand column, last paragraph - page 1914, right-hand column, last paragraph ---	1-4
A	VARLEY, ALAN W. ET AL: "Inflammation-induced recombinant protein expression in vivo using promoters from acute-phase protein genes" PROC. NATL. ACAD. SCI. U. S. A. (1995), 92(12), 5346-50 CODEN: PNASA6;ISSN: 0027-8424, 1995, XP002039443 see abstract see page 5346, right-hand column, paragraph 2 - paragraph 3 see page 5347, right-hand column, paragraph 4 - page 5348, left-hand column, paragraph 1 see page 5349, right-hand column, paragraph 2 - page 5350, left-hand column, paragraph 2 see page 5350, left-hand column, last paragraph - right-hand column, paragraph 1 ---	1-9
A	BETTS J C ET AL: "The role of NF-kappa B and NF-IL6 transactivating factors in the synergistic activation of human serum amyloid A gene expression by interleukin-1 and interleukin-6." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 268, no. 34, 5 December 1993, MD US, pages 25624-25631, XP002071439 see abstract see page 25624, right-hand column, paragraph 3 - page 25630, right-hand column, paragraph 3 --- -/--	1-9

1

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/EP 98/01363

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	RAY, ALPANA ET AL: "Analysis of the promoter element of the serum amyloid A gene and its interaction with constitutive and inducible nuclear factors from rabbit liver" GENE EXPRESSION (1993), 3(2), 151-62 CODEN: GEEXEJ;ISSN: 1052-2166, 1993, XP002039445 see abstract see page 152, left-hand column, paragraph 2 see page 154, left-hand column, paragraph 3 - page 155, left-hand column, paragraph 1 see page 159, right-hand column, paragraph 2 - page 160, left-hand column, paragraph 1 ---	1-9
A	ALPANA RAY ET AL.: "A novel cis-acting element is essential for cytokine-mediated transcriptional induction of the Serum Amyloid A gene in nonhepatic cells" MOLECULAR AND CELLULAR BIOLOGY, vol. 16, no. 4, April 1996, pages 1584-1594, XP002071440 see page 1584, right-hand column, paragraph 2 - page 1585, left-hand column, paragraph 1 see page 1585, right-hand column, paragraph 6 - page 1586, right-hand column, paragraph 2 see page 1591, left-hand column, paragraph 3 - page 1593, left-hand column, paragraph 3 ---	1-9
A	LI X ET AL: "Cooperative effects of C/EBP-like and NF kappa B-like binding sites on rat serum amyloid A1 gene expression in liver cells." NUCLEIC ACIDS RESEARCH, vol. 20, no. 18, 25 September 1992, OXFORD GB, pages 4765-4772, XP002071441 see abstract see page 4766, left-hand column, paragraph 2 - paragraph 3 see page 4767, left-hand column, last paragraph - page 4772, left-hand column, paragraph 2 --- -/--	1-9

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter ›nal Application No
PCT/EP 98/01363

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	BUTLER, A. ET AL: "Structure of the mouse serum amyloid A 5 (Saa5) gene: relationship to other members of the serum amyloid A family" SCAND. J. IMMUNOL. (1997), 45(2), 160-165 CODEN: SJIMAX;ISSN: 0300-9475, 1997, XP002039447 see abstract see page 160, left-hand column, paragraph 2 - right-hand column, paragraph 2 see page 161, right-hand column, last paragraph - page 162, left-hand column, paragraph 1 see page 162, right-hand column, paragraph 2 - page 164, left-hand column, paragraph 1 ---	1-4
A	US 5 151 508 A (JOHANNES M.H. SALBAUM ET AL.) 29 September 1992 see column 1, line 11 - line 19 see column 3, line 29 - column 4, line 54 ---	1-5
A	PATRICIA WOO ET AL.: "Structure of a human serum amyloid A gene and modulation of its expression in transfected L cells" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 262, no. 32, 15 November 1987, MD US, pages 15790-15795, XP002071442 see page 15794, left-hand column, paragraph 2 - right-hand column, paragraph 1 see page 15793, left-hand column, paragraph 3 see page 15791, left-hand column, last paragraph - right-hand column, paragraph 1; figure 2 see abstract ---	5-9
P,X	CLARISSA M. UHLAR ET AL.: "Use of the acute phase serum amyloid A2 (SAA2) gene promoter in the analysis of pro- and anti-inflammatory mediators: differential kinetics of SAA2 promoter induction by IL-1beta and TNF-alpha compared to Il-6" JOURNAL OF IMMUNOLOGICAL METHODS, vol. 203, no. 2, 25 April 1997, pages 123-130, XP004059576 see abstract see page 124, left-hand column, paragraph 3 - right-hand column, paragraph 2 see page 125, right-hand column, paragraph 3 - page 130, left-hand column, paragraph 2; figure 1 -----	1-5,7

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 98/01363

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 1, 2 and 4 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ›nal Application No
PCT/EP 98/01363

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5151508 A	29-09-1992	JP 3004794 A	10-01-1991